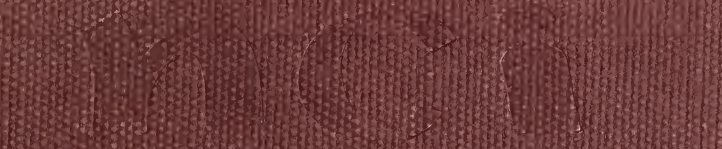


**International Symposium
Enzymatic Aspects of
Metabolic Regulation**



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International Symposium Enzymatic Aspects of Metabolic Regulation

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INTERNATIONAL SYMPOSIUM

ON ENZYMATIC ASPECTS OF

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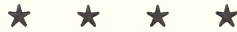
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1964

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1965

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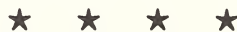


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FOREWORD

Important developments in biochemistry have taken place in Latin America, especially during the last 10 years. These have had a profound effect on the teaching and development of several areas in biochemistry. For this reason the 1966 Latin American symposium was held in Mexico City. The subject of the symposium, "Enzymatic Aspects of Metabolic Regulation," was a most appropriate one because of the large number of Latin American biochemists who are active in this area.

The program was planned under the leadership of Dr. P. P. Cohen, Department of Physiological Chemistry, University of Wisconsin; Dr. José Laguna, Facultad de Medicina, Universidad Nacional Autónoma de México; Dr. Guillermo Soberón, Instituto de Estudios Médicos y Biológicos, Universidad Nacional Autónoma de México; Dr. Hermann Niemeyer, Instituto de Química Fisiológica y Patológica, Facultad de Medicina, Universidad de Chile; and Francis T. Kenney, G. David Novelli, and Alexander Hollaender, Oak Ridge National Laboratory, Oak Ridge, Tennessee. The program was actually developed during discussions which were held on the occasion of the Gatlinburg conferences sponsored by the Biology Division of the Oak Ridge National Laboratory. It became obvious during these discussions that there was considerable strength in the field of biochemistry in Latin America and that the time was propitious for holding such a symposium.

Institutions in Mexico City have been active in developing in biochemical areas, and the Mexican groups were responsible for the organizing and handling of the entire symposium. Special thanks for support should be given to the U.S. Atomic Energy Commission, the National Science Foundation, and especially the Ford Foundation, which has now made funds available to broaden these symposia. The National Cancer Institute generously made this symposium, as it had done for some of the previous symposia, part of their Cancer Monograph Series. Local arrangements for the Symposium were most efficient, and the generosity of the industrial contributors made the meeting an especially pleasant one.

Dr. Laguna, as President of the Organizing Committee of the Symposium, made the opening remarks. Then Dr. P. P. Cohen went over the development of biochemistry in Latin America—particularly in Mexico, where the first biochemical society in Latin America was organized. Dr. Guillermo Massieu, Director of the Instituto Politécnico Nacional, gave a short talk, and finally Dr. Javier Barros Sierra, Rector of the University of Mexico, spoke.

Those from outside Mexico who attended the conference were very impressed by the country's remarkable restoration of her cultural history. Opportunities were abundant for members of the Symposium to see the artifacts of Mexico's past and thus get the proper background for an understanding of her modern development.

ALEXANDER HOLLAENDER

INTRODUCTION

The International Symposium on Enzymatic Aspects of Metabolic Regulation, held in Mexico City from November 28 to December 1, 1966, was a success of the highest significance for the group of Latin American biochemists. This Symposium is the sixth in a series of annual meetings held in different capital cities of Latin America. It was jointly organized under the auspices of the Biology Division of the Oak Ridge National Laboratory, Oak Ridge, Tennessee, and other governmental agencies of the United States, the Ford Foundation, the University of Wisconsin, and the universities from the host country. However, on this occasion, more than ever, the Latin American scientists made important contributions. Indeed, the opening address was delivered by Dr. Luis F. Leloir, and 16 of the 26 papers were original contributions of international significance given by biochemists from Argentina, Chile, Brazil, and Mexico. This circumstance reflects the fact that the topic of metabolic regulation has been pursued with a great deal of interest and efficiency by several investigators of these countries. Most of the other contributors and the moderators were American scientists, although some Europeans also participated.

The adequate judgment and persuasiveness of Dr. Philip P. Cohen represented the important role of the University of Wisconsin in the organization of the Symposium. The Universidad Nacional Autónoma de México and the Instituto Politécnico Nacional also made every effort that was necessary for the arrangements for the Symposium. Important aid came from the Biology Division of the Oak Ridge National Laboratory, whose former Director, Dr. Alexander Hollaender, very actively worked in the arrangements for the meeting. The expenses were met through donations from the Universidad de México, the Instituto Politécnico Nacional, and the U.S. Public Health Service, U.S. National Science Foundation, and U.S. Atomic Energy Commission, as well as the Ford Foundation, and several pharmaceutical companies.

A rather favorable circumstance permitted the selection of Mexico as the host country for this Symposium, namely, the fact that a proper biochemical environment has been built up during the last decade. The spirit of collaboration, highly developed among the different Mexican groups, within and outside of our institutions, very effectively guaranteed the necessary backbone for continuous progress of the biochemical sciences in our country. It is enough to say, in this respect, that the Mexican Society of Biochemistry which will soon be 10 years old and is perhaps the oldest organization of biochemists in the Latin American countries, has about 50 members, most of them independent investigators, whose scientific works appear regularly in international journals of biochemistry. Several factors have contributed to the development of biochemistry in Mexico; among them is the constructive attitude of those devoted to science as well as the authorities at the institutional and governmental level. All of these persons have generously contributed to the establishment of research groups, in the provision of laboratory space, equipment, and the promotion and recruitment of personnel. This has made possible the consolidation of the biochemical sciences in the Universidad Nacional, the Instituto Politécnico Nacional, medical organizations of the Ministry of Public Health, and the Mexican Institute of Social Security. Moreover, there are clear signs that industrial and philanthropic organizations have started to support activities in this field with the bestowal of endowments and legacies. Other agencies eventually may also collaborate to sustain the joint effort at a high level.

Another important aspect of the favorable atmosphere prevailing in this country, which has given solid support to our achievements, is the existence of several programs for the training of graduate students, two at the Instituto Politécnico Nacional and one at the Universidad Nacional Autónoma de México. These programs include at the present time about 60 graduate students. The courses are oriented to provide academic preparation and practical instruction that will enable the students to fulfill their future tasks efficiently, either in scientific research or in the direct application of their knowledge to problems of industry, agriculture, and medicine. The professors in charge of the programs were trained at first-rate laboratories in the United States and Europe. It seems, therefore, that very fortunately Mexican

biochemistry is beginning to have an international and universal character which is quite appropriate for the training of young people interested in the field. The continuing advice of Dr. Cohen has been of great meaning in the development of Mexican biochemistry.

It is a great satisfaction to recall the encouraging words from participants in the Symposium, especially those from our American and European colleagues. They pointed out that the participation in comments and discussions of the investigators and students was impressive and reflected significant progress.

We cannot do less than recognize the extraordinary and enthusiastic collaboration of Dr. Philip P. Cohen and Dr. Alexander Hollaender who gathered a select group of outstanding scientists to participate in the Symposium and moderate the discussions. Of great value was the help of the other members of the organizing committee, Drs. Kenney, Volkin, and Novelli of Oak Ridge, and that of our Chilean colleague Dr. Herman Niemeyer.

The fruits of the Symposium were not long in showing up. Along this line we can say that, independently of the gains in the scientific field of metabolic regulation, there were spectacular and splendid gains in the field of human relations. Also, an *ad hoc* Committee of Latin American Scientists for the Biochemical Sciences was created, which should be of great service in furthering our activities. We look forward to close ties which will undoubtedly strengthen friendship and understanding among men of science, as well as among our countries.

Dr. José Laguna
Jefe del Departamento de Bioquímica
Facultad de Medicina
Universidad Nacional Autónoma de México

Dr. Guillermo Soberón
Director del Instituto de Estudios Médicos
y Biológicos
Universidad Nacional Autónoma de México

MONDAY MORNING

***Chairman:* Guillermo Massieu**

Regulation of Glycogen Metabolism^{1,2}

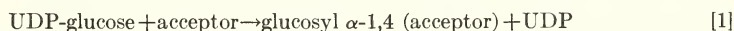
LUIS F. LELOIR, *Instituto de Investigaciones Bioquímicas, Fundación Campomar, y Facultad de Ciencias Exactas y Naturales, Buenos Aires 28, Argentina*

THE REGULATION of glycogen metabolism is intimately associated with the maintenance of the normal level of blood sugar and is therefore of considerable physiological and medical interest. Some of the enzymes involved have been studied in great detail, and it has been found that they are transformed from inactive to active forms under the influence of various physiological factors (1).

SYNTHESIS OF GLYCOGEN

Before discussing the regulation it may be appropriate to give a brief survey of the pathways and enzymes of glycogen metabolism.

Synthesis is believed to be carried out by transfer of glucose residues from UDP-glucose to an acceptor, forming an α -1,4 glucosidic bond as follows:

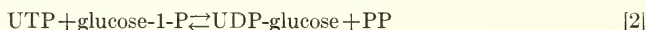


The enzyme involved is glycogen synthetase (2-4). The acceptor may be a preexisting glycogen molecule, but smaller fragments such as maltotriose and maltotetraose (5) or a branched maltopentaose (6) can also be used but at a lower rate. Glycogen is used 500 times faster than maltotetraose and 5,000 times faster than maltotriose. By successive transfers from UDP-glucose, 10-12-unit chains of α -1,4 linked glucose units are formed, and these can serve as substrates for the branching enzyme. This enzyme catalyzes the transfer of 6 or more unit sections (7), which become joined α -1,6.

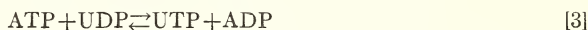
¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. O. Cori, p. 41.

The formation of UDP-glucose is catalyzed by UDP-glucose pyrophosphorylase according to the following reaction:



In the glycogen synthetase reaction, UDP is formed. It can be phosphorylated again to UTP and reused for synthesis. Phosphorylation is brought about by transfer from ATP, which in turn is formed by oxidative phosphorylation. The reaction is as follows:



DEGRADATION OF GLYCOGEN

Glycogen degradation occurs by removal of glucose residues from the nonreducing ends of the chains, the product being glucose-1-P. Phosphorylase action stops on reaching the branching points when the chains are shortened to about 4 units. A transfer of a segment of 3 or more units is catalyzed by oligoglucan transferase (8). The product is a linear chain bearing a single glucose unit linked α -1,6. This residue may be hydrolyzed, giving free glucose, by α -1,6 glucosidase. These two enzymes, the transferase and glucosidase, seem to be intimately associated.

Once the α -1,6 linkage has been removed as described, the remaining chain can be degraded further by phosphorylase and the whole process is repeated on reaching a new branch point.

KEY ENZYMES

Regulation of glycogen metabolism is carried out mainly by changes in the activity of two enzymes. These are phosphorylase and glycogen synthetase, which catalyze glycogen degradation and synthesis, respectively. It seems that regulation is carried out by changing the activity of these enzymes but in opposite directions. For instance, degradation is produced by an increase in the activity of phosphorylase or by a decrease in synthetase, and the inverse occurs during synthesis. The activities of the other enzymes involved in glycogen metabolism are believed to be in excess and presumably remain constant when the two key enzymes change.

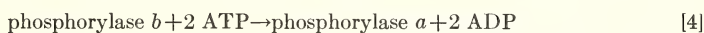
A common property of the two key enzymes is that they appear under different forms which are specifically activated by certain substances.

Muscle phosphorylase was found by Cori *et al.* (9) to be activated very specifically by AMP, and since then it has been suspected that this activation is important in the regulation of glycogen metabolism.

Of course, at the time, this was difficult to understand because phosphorylase was believed to be responsible both for the synthesis and degradation of glycogen. Activation of glycogen synthetase by glucose-6-P was detected with the muscle (2) and liver (3) enzymes. Here again the effect is very specific. Only glucosamine-6-P and galactose-6-P were found to have some activity. Another compound which has been found to be active, but is not known to occur in tissues, is 1,5-sorbitan-6-P (10).

The two enzymes are known to occur under forms which differ in the effect of the activator. The reversible transformation of muscle phosphorylase from the form dependent on AMP (*b* form) to independent (*a* form) has been the object of very fine studies (11).

The reaction:



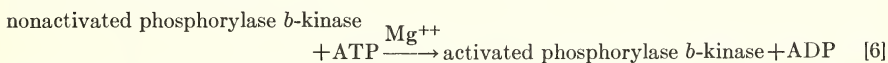
is a phosphorylation of serine residues in the enzyme. Since phosphorylase *a* dimerizes in concentrated solution (12-14), the reaction is often written as follows:



Several of the amino acids around the serine residue to which the phosphate becomes attached have been identified (15).

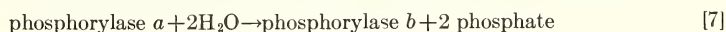
Some of the properties of muscle and liver phosphorylase are different. The amino acids joined to the serine phosphate residue are not the same for the rabbit liver and muscle enzymes (16). Inactive liver phosphorylase does not become active even in the presence of AMP.

The enzyme that catalyzes muscle phosphorylase activation (reaction [4]) is called phosphorylase *b*-kinase. It occurs also in 2 forms which are referred to as nonactivated and activated. The conversion of one into the other



appears to be like reaction [4], a transfer of phosphate from ATP, and is accelerated by cyclic 3',5'-AMP.

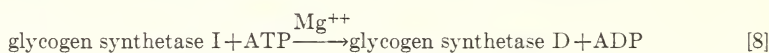
The reversion of phosphorylase *a* to *b* may be written as follows:



This reaction is catalyzed by a very specific phosphatase. Of many substrates tested, only the serine phosphate peptide obtained by degradation of phosphorylase is hydrolyzed, but much more slowly (15) than the phosphorylase *a* substrate.

Knowledge on glycogen synthetase has not progressed as much as that on phosphorylase. The reasons are that the enzyme was discovered more recently and that it is not as easy to purify. The different forms of synthetase and the conversion reaction have been studied by Traut, Larner, and others. The reactions are similar to those of phosphorylase,

but in the opposite direction. That is, when phosphorylase is activated, synthetase is deactivated. The transformation of the glucose-6-P independent form to the dependent form can be written as follows:



It may be observed that this reaction is similar to the phosphorylase *b* to *a* conversion (reaction [4]) and to the nonactivated to activated transformation of phosphorylase *b*-kinase (reaction [6]).

The I to D conversion has been carried out with ^{32}P -labeled ATP. The label was recovered in a serine phosphate, and curiously enough the amino acids around the serine were found to be the same as in phosphorylase (17). The sequence found was: (Arg Lys)-Glu-Ile-Ser-P-Val-Arg. The two enzymes are also similar in their specificity for the polysaccharide.

REGULATION BY METABOLITES

As a consequence of the studies on the interconversion of phosphorylases, the major role in regulation was attributed to such changes and AMP activation was relegated to the background. However, other workers still believed that activation by AMP and glucose-6-P might have a direct role in regulation, and that it was not adopted by cells only to permit biochemists to distinguish two types of enzymes. These activation phenomena surely have a more profound meaning in the complex mechanism of metabolic regulation. An increase in AMP may be translated jocosely as a message to phosphorylase *b*, reading: "phosphorylating reactions running down. Provide more carbohydrate." Similarly an increase in glucose-6-P would be read by glycogen synthetase as follows: "Plenty of carbohydrate available, proceed to glycogen synthesis." The regulation of phosphorylase by AMP may be, according to Atkinson and co-workers (18, 19), an ancestral pattern of intracellular control; and the control by cyclic 3',5'-AMP may have evolved from it "as a means by which metabolic processes in complex organisms can be controlled by present or anticipated needs of the whole organism rather than of the single cell." In relation to their hypothesis, it would be interesting to know the distribution of AMP-activated phosphorylase and of cyclic 3',5'-AMP in nature. In some instances, as in plants, phosphorylase is known not to be activated by AMP.

Besides activation by AMP, other substances influence activity. According to Morgan and Parmeggiani (20, 21), regulation of phosphorylase *b* activity is modified not simply by the concentration of AMP but by the ratio of ATP/AMP + glucose-6-P.

The concentration of inorganic phosphate is also important. Changes of this type are assumed to be responsible for glycogenolysis in heart during anoxia (20, 21). According to Helmreich *et al.* (22), the contraction in the frog sartorius muscle produces only a small change in AMP, but ATP decreases and ADP increases. They observed that ADP inhibits phosphorylase *b* like ATP. The ratio of inhibitor to activators is then $(\text{ATP} + \text{ADP})/\text{AMP}$ and it does not change by contraction. Helmreich *et al.* (22) concluded that it is unlikely that changes in adenine nucleotides are responsible for the changes in activity of phosphorylase *b*.

Anoxia in liver produces glycogenolysis without activation of phosphorylase (23). In this case, activation by AMP does not explain the results because inactive liver phosphorylase (which corresponds to *b* of muscle) is not activated by it. A possibility is that the rate of glycogenolysis does not change, but that synthesis is stopped due to lack of phosphorylating reactions necessary for UDP-glucose formation. Other effects of nucleotides which may be important are: the inhibition of phosphorylase *b*-kinase by excess ATP (24) and the inhibition of phosphorylase phosphatase by AMP (concentrations as low as 5×10^{-6} M are effective) (25).

Changes in glucose-6-P have been found in many experiments, but their importance is doubtful since special precautions are necessary in order to measure real values. For instance, Hornbrook *et al.* (26) observed that in brain 1 minute of ischemia is sufficient to raise glucose-6-P concentration to more than double. Young (27) reached similar conclusions after studies on liver. Therefore many of the changes of glucose-6-P which have been described will have to be reinvestigated. These include the action of insulin and corticoids.

UDP-glucose concentration may also have a role in regulation since it has been found that it inhibits phosphorylase (28).

Another substance which affects the activity of the key enzymes is glycogen, and it has been assumed that glycogen is involved in its own regulation. The mechanism would be such that large increases in glycogen would favor its own destruction or decrease synthesis. The evidence is not very solid. Danforth (29) found in muscle an inverse relationship between glycogen content and the ratio glycogen synthetase I/total glycogen synthetase. Similar results were obtained with HeLa cells (30), that is, as if an increase in glycogen produced inactivation of glycogen synthetase. Other effects of glycogen which have been described are the inhibition of glycogen synthetase D phosphatase, a stimulation of phosphorylase *b*-kinase and a decrease in the K_m for phosphorylase *b* of nonactivated phosphorylase *b*-kinase (24). These two effects would tend to increase phosphorolysis of glycogen. It may be mentioned (31) that phosphorylase *a* can be activated up to 40-fold

by AMP when the concentrations of glycogen, inorganic phosphate, and glucose-1-P are very low. Both the *a* and *b* forms of phosphorylase are therefore regulated by AMP concentration, but the *b* to *a* conversion leads to greater sensitivity to low levels of AMP.

NERVOUS REGULATION

The study of nervous regulation may be traced back to Claude Bernard. He not only discovered glycogen, but observed in 1855 that stimulation of the floor of the fourth ventricle increased blood sugar and produced glycosuria. Evidently he was the first to observe the result of glycogenolysis induced by nervous action. It is due partly to the secretion of adrenalin by the adrenals, as was proved by Houssay and Molinelli (32). However, it still occurs after removing the adrenals, so there may be a direct action on the liver.

Modern work has shown that the adrenergic system is spread throughout the brain and that it is involved in many functions in the rest of the body. The impulses arising from the brain may cause release of catecholamines which are known to act on phosphorylase and glycogen synthetase causing glycogenolysis.

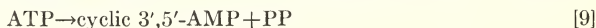
That there can be a direct action has been proved by Shimazu and Fukuda (33), who reported that stimulation of the splanchnic nerves in rabbits produces an increase in active phosphorylase and in glucose-6-P phosphatase and that the increases still occur after adrenalectomy or pancreatectomy.

HORMONAL REGULATION

Several hormones are known to influence glycogen metabolism. Adrenalin and glucagon produce increased blood sugar and glycogenolysis. Insulin leads to hypoglycemia and increased muscle glycogen. Glucocorticoids raise liver glycogen. Investigations on the activity of different enzymes after treatment with hormones have shown some changes which are summarized in table 1 and commented on in more detail below.

Adrenalin and Glucagon

The mechanism by which adrenalin and glucagon produce phosphorylase activation has been expertly reviewed (34), so that only a brief mention is necessary. These substances act on cell membranes and produce activation of AMP cyclase which catalyzes the reaction



The interaction between glucagon and adrenalin with the receptor in the cell membrane and how this affects cyclase are still a mystery.

TABLE 1.—Action of hormones on enzymes*

Enzyme	Insulin	Adrenalin	Glucagon	Corticoids
Liver glycogen synthetase.....	+	—	—	+
Muscle glycogen synthetase.....	+	—	0	—
Liver phosphorylase.....	—	+	+	0
Muscle phosphorylase.....	—	+	0	0

* The signs represent: +, increase; —, decrease; 0, no action.

Besides accelerating the *b-a* transformation of phosphorylase, cyclic 3',5'-AMP acts on glycogen synthetase. It was found by Belocopitow (35) that, in rat diaphragm, adrenalin produces a decrease in total glycogen synthetase, and later work showed that cyclic 3',5'-AMP accelerates the conversion of I to D glycogen synthetase (36, 37).

Glucagon is 10–100 times more active than adrenalin as a glycogenolytic agent in liver (23, 38), and therefore it has been concluded that glucagon is responsible for the normal regulation of blood sugar. In agreement with this idea, it has been found that decreases in blood sugar lead to increased glucagon secretion (39).

In skeletal muscle, glucagon does not produce glycogen breakdown, but it is active on heart muscle.

Insulin

It seems well proved that insulin increases the penetration of glucose and also glycogen deposition in muscle.

Many studies have been done in which the enzymes of glycogen metabolism have been measured after insulin had been given. No change has been detected in phosphorylase, UDP-glucose pyrophosphorylase, or phosphoglucomutase. Reports on an elevation of glucose-6-P level (40) were not confirmed. A definite increase in glycogen synthetase I was first detected by Lerner and co-workers (41, 42) in rat diaphragm and it has been confirmed by others. The increases produced by I are about 30%. In liver of diabetic rats, Steiner (43) observed, after insulin injection, an increase in glycogen synthetase which appeared after 40 minutes and lasted about 6 hours.

Steiner (43) also did experiments on normal, fasted rats. Feeding produced an increase in glycogen synthetase mainly in the I form. Glycogen increased markedly. Treatment with anti-insulin serum nearly suppressed these changes, whereas normal serum had no effect. It may be concluded that the rise in blood sugar by itself is not suffi-

cient for increasing glycogen deposition and that insulin secretion is necessary for this effect.

In HeLa cells, Alpers (30) found that insulin increases the I form of glycogen synthetase about 30% but that glycogen decreased.

Corticoids

It has been known for a long time that glucocorticoids lead to increased formation of carbohydrate from amino acids. They also increase glycogen content in liver and muscle. The changes produced by corticoid administration have been studied by several workers. Tarnowski *et al.* (44) found that total glycogen synthetase increased about 50% and concomitantly UDP-glucose decreased. No changes were detectable on phosphorylase, UDP-glucose pyrophosphorylase, phosphoglucumutase, or glucose-6-P phosphatase.

Liver glycogen synthetase of adrenalectomized rats was found to be mostly in the D form and treatment with hydrocortisone led to transformation to the I form (45). Fasting reduces total synthetase in liver and cortisol raises it back to normal values.

Increases in glucose-6-P have also been reported (46, 47) but since this substance increases very rapidly post mortem, the value of these results is doubtful.

Besides increasing glycogen formation, cortisol seems to decrease degradation. Thus, Von Holt and Fister (48) observed that, after allowing liver glycogen to incorporate radioactivity from ^{14}C -pyruvate, the decrease in radioactivity was slower in cortisol-treated animals than in the controls.

GLYCOGENOLYSIS DURING MUSCLE CONTRACTION

Excitation of the nerves of muscle, contraction, and glycogenolysis are three interconnected phenomena. What are the factors that start glycogen breakdown and lactic acid formation? It has been suspected for years that the *b* to *a* transformation of phosphorylase is important. A study of the problem has been done in rats with lack of phosphorylase *b*-kinase. In these rats it was observed by Danforth and Lyon (49) that during tetanic contraction no phosphorylase *a* is formed. The increase in lactate was slower than in normal muscles and the final amount was only about half. These experiments prove that the *b* to *a* transformation is of some importance, but not indispensable, and that some other mechanism may lead to increased glycogenolysis.

Some studies related to the role of catecholamines and cyclic 3',5'-AMP have been done. It seems that the mechanism of *b* to *a* transformation during muscle contraction may differ from that of adrenalin.

In frog sartorius muscle, Danforth *et al.* (50) found that isometric contraction leads to *b*→*a* transformation with a half time of activation of 0.7 seconds. Injection of adrenalin produced activation, but with a much longer half time (370 seconds). The two mechanisms could be distinguished with dichloroisoproterenol, which inhibited the effect of adrenalin but not that of contraction. *In vivo* experiments (51) also revealed differences between the action of electrical stimulation and of adrenalin. Phosphorylase *a* increased in both cases, but cyclic 3',5'-AMP increased only after adrenalin.

A mechanism of producing glycogenolysis which may be of importance in relation to muscle contraction is one in which calcium ions are involved. It was observed by Meyer *et al.* (52) that calcium ions together with a specific protein can produce a conversion of nonactivated phosphorylase *b*-kinase.

A similar effect was detected on the I→D conversion of glycogen synthetase (36, 53). The hypothesis that an increase in calcium ion concentration produces glycogenolysis is attractive because these ions are believed to be involved in muscle contraction (54). However, some points are not clear. The calcium concentration that produces transformation of phosphorylase *b*-kinase and glycogen synthetase is about 10^{-3} M, whereas concentrations which are believed to play a role in muscle contraction are about 10^{-6} M. Furthermore, it is not known how the enzymes activated by the calcium mechanism can be reverted to the original form. The D form of glycogen synthetase obtained with calcium ions seems to be different from that formed by the ATP mechanism (55).

Finally, it is possible that the specific protein is a calcium-activated protease and that these changes have no physiological importance. In fact, similar changes can be produced with trypsin. Attempts to separate the protein factor required for phosphorylase and glycogen synthetase have been negative (36).

FEEDING AND FASTING

Why does glycogen synthesis increase after food intake? The sequence of events in liver may be as follows: The increase in blood sugar concentration in portal blood and in liver cells would result in an increase of glucose phosphorylation. The rate of the following reaction



is proportional to glucose concentration in blood since the K_m of glucokinase for glucose (about 10 mM) is similar to the concentration

in blood. This would result in an increase of glucose-6-P concentration and therefore in a stimulation of glycogen synthetase (27).

After long fasting periods, the changes found after glucose administration were different. Steiner (43) found that glucose-6-P did not increase, but that glycogen synthetase (especially the I form) and glucokinase did increase. Insulin is known to be secreted after glucose injection (56), so surely this hormone is involved in these changes.

During fasting, glycogen has to be broken down to maintain blood sugar level. Phosphorylase has to be more active than after feeding. It is likely that the change would be brought about by glucagon secretion, but we do not know how this secretion is regulated. There must be some mechanism which is sensitive to changes in blood sugar concentration and sends orders to the liver enzymes.

CHANGES IN GLYCOGEN STRUCTURE

In addition to the amount of glycogen in tissues, there is another aspect to be considered. This is the structure and the molecular weight distribution.

Abnormal glycogens are found in cases of glycogenosis. For instance in Type II, which is attributed to the lack of amylo 1,6-glucosidase, the glycogen has short exterior branches. In Type IV, where the branching enzyme is deficient, the exterior branches are longer than normal.

Another aspect that has been studied is the molecular weight distribution and in particular that of liver glycogen. Extraction by mild methods yields glycogen of very high molecular weight. Glycogen of equally high molecular weight, which has a similar aspect when examined under the electron microscope, has been prepared *in vitro* with purified phosphorylase and branching enzyme (57). However, the natural samples differ from those prepared *in vitro* in that they are more labile. Native glycogen is broken down by acid, alkali, and heat to molecules of 8 million molecular weight. Under the same conditions the samples prepared *in vitro* also break down, but to a lesser extent and as if the molecules broke down successively in halves. It seems that high molecular weight liver glycogen has some links that are more labile than the others due to the presence of some different type of bond or to some conformational factor.

The changes in molecular weight distribution under different conditions have been studied by Bueding and Orrell (58). They found that in *Fasciola hepatica* the high molecular weight component disappeared during fasting and reappeared after feeding glucose. In *Hymenolepis diminuta* (59) the mean molecular weight was higher when glycogen content increased. In rats, Mordoh *et al.* (60) found that the molecular

weight distribution of liver glycogen was rather variable, but they could not find out which factors affect it. A more thorough study was done in mice by Parodi (61). Only a slight decrease in molecular weight was found to take place under the influence of factors which decrease liver glycogen, such as fasting, epinephrine, glucagon, insulin, and ischemia. After glucose administration, glycogen was increased but the molecular weight distribution remained unchanged. It also remained unchanged after administration of glucocorticoids, growth hormone, and various monosaccharides.

The constancy of the molecular weight distribution requires a delicate mechanism of regulation. For instance, to maintain the same distribution during synthesis, the mechanism of formation of new molecules must act in proportion to the rate of addition of glucose units and of branching.

OTHER PROBLEMS

We believe we know the enzymes involved in the formation and breakdown of glycogen. The properties of these enzymes and the action of activators and inhibitors have been well studied. All this information can be put in the form of equations and calculated with a computer. A study of this type has been published by London (62). There remain, however, many aspects which are not clear. The role of some enzymes is still to be determined although a reasonable hypothesis can be proposed. A lysosomal glucosidase with an optimum pH at 5 is found in liver and probably other tissues (63). It hydrolyzes maltose about 6 times faster than glycogen. According to Baudhuin *et al.* (64) this enzyme is lacking in a Type II glycogenosis (Pompe's disease), with the result that lysosomes become packed with glycogen and are enlarged. Presumably they grow so much that the cells are damaged. It would appear that normally glycogen penetrates into the lysosomes and is broken down by the acid glucosidase.

Another glucosidase, with an optimum at pH 6, is present in the cytoplasm (63); it acts about 25-fold faster on maltose than on glycogen and also catalyzes transglucosylations as was described by Stetten (65). The role of neutral glucosidase might be to hydrolyze abnormal glucosides which might reach the liver cells, or it might be to break down malto-oligosaccharides formed by α -amylase. The greater part of the malto-oligosaccharides, which have been described as possible intermediates in glycogen breakdown, appears to be formed mainly as an artifact after homogenization of the liver. If α -amylase is inhibited as with HgCl_2 , hardly any oligosaccharides are detectable (60, 66). This, however, does not exclude the possibility that malto-oligosac-

charides are formed in small quantities and either built up to new glycogen molecules or destroyed by glucosidase.

Another aspect of glycogen metabolism on which we have only scanty information is that related to cell structure. The parts of the liver cells that contain glycogen seem to be the same as those located where the tubular endoplasmic reticulum is found. This fact was first observed by Porter and Bruni (67).

In the fetus, the smooth endoplasmic reticulum was absent when glycogen was abundant in the late gestation period. This became evident after birth when glycogen declined (68). It was concluded, therefore, that smooth endoplasmic reticulum is related to glycogenolysis. The problem has been studied by several workers (69, 70) and is not clear. The enzymes that are localized in the endoplasmic reticulum are glucose-6-P phosphatase (71) and probably amylase. Glycogen is not only unevenly distributed within each cell but also in different parts of the liver lobuli. In some cases there are peripheral deposits and in others only the central zones contain glycogen. According to Ekman and Holmgren (72), after fasted mice are fed, glycogen appears in the peripheral cells which are the first irrigated by the portal blood and those that presumably receive the highest concentration of sugar and hormones. Two hours after feeding, glycogen appeared in the central part of the lobuli, and after 5 hours it was deposited evenly in different parts.

Regulation of glycogen includes, therefore, several aspects: 1) the soluble enzymes with their different forms and their activators and inhibitors, 2) the localization of the enzymes within certain regions of the cell, and 3) the different activity of individual cells within an organ. All these aspects will have to be investigated in order to obtain a clearer view of the regulation of glycogen metabolism.

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Allosteric Properties of Yeast Glycogen Synthetase^{1,2,3,4}

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SUMMARY

Yeast glycogen synthetase is activated by glucose-6-phosphate. Several anionic substances (chloride, maleate, etc.) inhibit the enzyme in the absence of glucose-6-phosphate. Addition of glucose-6-phosphate restores much of the original activity. When chloride was used as model effector, inhibition was partially competitive toward UDP-glucose. Plots of activity versus UDP-glucose concentration are hyperbolic in the absence or presence of glucose-6-phosphate or chloride. When activity is represented as a function of glucose-6-phosphate concentration, sigmoid curves are obtained in the presence of chloride. Variation of chloride at fixed concentration of glucose-6-phosphate also gives rise to sigmoid curves. Treatment of the enzyme with 2,4-dinitrofluorobenzene at pH 8 leads to irreversible loss of sensitivity to chloride, while most of the activity remains. It

is concluded that inhibitors bind to a site different from that of substrate. A study of inhibition at different pH values showed that some substances inhibit more strongly at pH 6, the presumed physiological value for yeast, than at pH 7.5. The most effective inhibitors are the nucleotides ADP, ATP, and GTP. Glucose-6-phosphate also relieves this inhibition. A control mechanism of glycogen synthesis *in vivo* is envisaged, in which the concentration of glucose-6-phosphate, regulated through phosphofructokinase by the levels of ATP and AMP, would determine the rate of glycogen synthesis. An additional control by ammonium ions, acting through the same mechanism, would channel, when necessary, the carbon of glucose toward the nitrogenous compounds needed for growth.—*Nat Cancer Inst Monogr* 27: 19–28, 1967.

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² See Discussion of this paper conducted by Dr. O. Cori, p. 41.

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GLYCOGEN SYNTHETASES (uridine diphosphate D-glucose: glycogen α -4-glucosyltransferases) from different sources vary in their dependence on the activator, glucose-6-phosphate (glucose-6-P). The extreme cases reported are the D form from muscle (1), completely inactive in the absence of glucose-6-P; and the enzymes from locust muscle (2) and cockroach fat body (3), which are unaffected by the phosphoric ester. The yeast synthetase represents an intermediate case, showing a moderate effect of glucose-6-P, which markedly depends on pH (4).

Recent interest in the regulatory properties of enzymes (5) prompted us to explore the action of glucose-6-P. This study revealed that a number of anionic substances inhibit yeast synthetase and that glucose-6-P can reverse the inhibition.

The effect (table 1) is that glucose-6-P activates many times a previously inhibited enzyme, giving a much larger relative stimulation than in the absence of inhibitor. The effect was not simply one of ionic strength, since some anions were not inhibitory, while close analogs depressed enzymatic activity, as in the pairs succinate-maleate and lactate-pyruvate. The concentration at which the different substances were effective was generally high and physiologically insignificant.

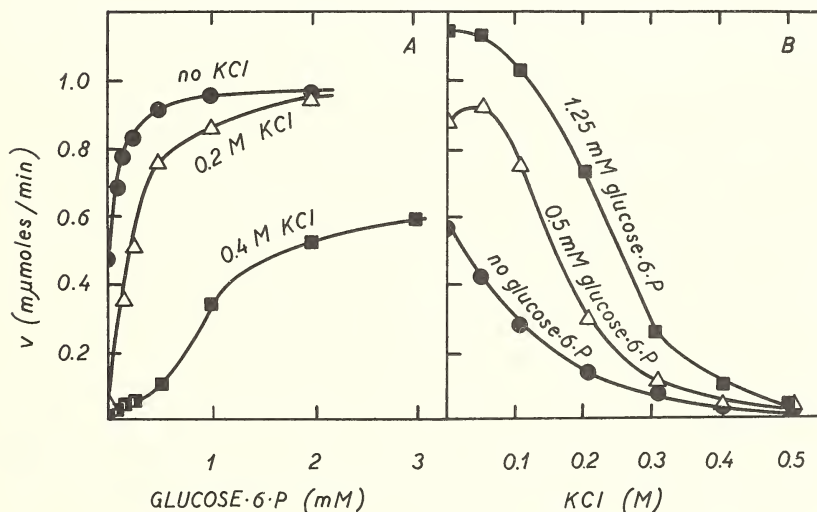
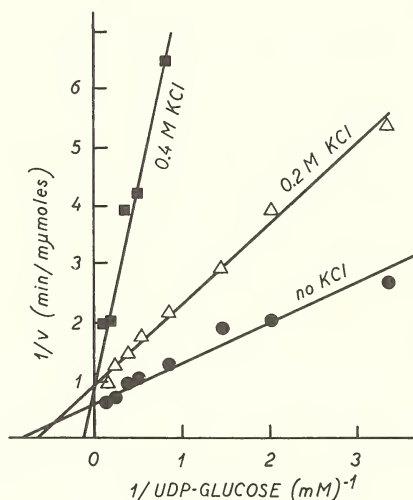
In further study of the effect, chloride was chosen as a typical modifier. Text-figure 1 shows that the inhibition is a mixed, partially com-

TABLE 1.—Inhibition of glycogen synthetase by different substances and reversion by glucose-6-phosphate

Additions to reaction mixture	Concentration (M)	Activity		Activation ratio + glucose-6-P — glucose-6-P
		— Glucose-6-P	+ Glucose-6-P	
None.....	—	100	172	1.72
Chloride.....	0.2	35	168	4.7
Nitrate.....	0.1	44	142	3.2
Sulfate.....	0.1	36	140	3.7
Phosphate.....	0.2	100	—	—
Maleate.....	0.2	19	170	7.9
Succinate.....	0.2	100	148	1.48
Pyruvate.....	0.2	68	170	2.7
Lactate.....	0.2	114	166	1.46

petitive type, and that rectilinear Lineweaver-Burk plots were obtained both in the absence and in the presence of chloride. On the other hand, graphical representation of activity against glucose-6-P concentration gave sigmoid curves (and curved Lineweaver-Burk plots) in the presence of the inhibitor, as shown in text-figure 2 A. The same occurred in the reciprocal situation, that is, when chloride was varied

TEXT-FIGURE 1.—Inverse plots of the rate as a function of UDP-glucose concentration in the absence and in the presence of chloride. Assay conditions as in table 1.



TEXT-FIGURE 2.—A. Effect of changing the concentration of glucose-6-phosphate, in the presence of different amounts of chloride. B. Effect of changing the concentration of chloride at fixed concentrations of glucose-6-phosphate. In both cases the assay conditions were as in table 1.

at fixed concentrations of glucose-6-P (text-fig. 2 *B*). These results resembled those obtained with several allosteric enzymes (6). Therefore, it was decided to investigate further whether uridine diphosphate glucose (UDP-glucose) and chloride bind to the same or different sites of the enzyme. Several attempts were made to destroy the hypothetical inhibitor site, *i.e.*, to desensitize the enzyme to chloride, by various treatments such as heating, adding urea, mercuric salts, or *p*-chloro-mercuribenzoate. All these conditions led either to no change or to irreversible inactivation of the enzyme.

The only procedure so far yielding satisfactory results is dinitrophenylation of the enzyme (7) in the presence of UDP-glucose at precisely determined values of *pH* and dinitrofluorobenzene concentration. As shown in table 2, the dinitrophenylated enzyme was no longer inhibited by chloride or maleate. The activation by glucose-6-P (not shown) and the inhibition by a true competitive inhibitor, such as UDP, were preserved.

TABLE 2.—Properties of dinitrophenylated enzyme

The dinitrophenylation mixture contained 25 mM Tris-succinate buffer at *pH* 8.5, 12.5 mM UDP-glucose, 1.5 mM 2,4-dinitrofluorobenzene, and enzyme. The final *pH* of the mixture was 8. The mixture was shaken for 15 minutes in the dark at room temperature, then cooled in ice; aliquots were withdrawn and added to the incubation mixture. The final composition of the incubation mixture was 0.1 M glycylglycine buffer, *pH* 7.5, 5 mM UDP-glucose-¹⁴C (specific activity 50,000 cpm/ μ mole), and 3% glycogen, in a total volume of 0.1 ml. Other components were added as indicated below. After 30 minutes of further incubation at 30 C, the labeled glucose incorporated into glycogen was measured as in table 1. After dinitrophenylation, 53% of the original activity was recovered.

Inhibitor added	Concentration (M)	Inhibition	
		Native enzyme (%)	Dinitrophenylated enzyme (%)
KCl.....	2×10^{-1}	45*	0
Maleate.....	2×10^{-1}	43*	2
UDP.....	5×10^{-4}	39	37
UDP.....	2×10^{-3}	67	69

*The inhibition is lower than in table 1 because of the higher concentration of UDP-glucose in this experiment.

A survey of substances that might affect the enzymatic activity showed that several of them, such as trehalose phosphate, 3-phosphoglyceric acid, and inorganic phosphate, all at 10 mM, had an activating effect similar to that of glucose-6-P. Even inorganic sulfate, which was inhibitory at high concentrations (*see* table 1), stimulated the activity at 10 mM. None of these compounds was, however, capable of reversing the inhibitory action of chloride. Kinetic analysis of the

activating effect of glucose-6-P at two different *pH* values showed that the phosphoric ester, in the absence of chloride, only increased the maximum velocity, whereas, as a reactivator of the inhibited enzyme, it affected both the maximum velocity and especially the K_m .

Earlier determinations of the *pH* activity curve, with Tris-maleate as buffer, had shown a rather sharp peak at *pH* 7.5, in the absence of glucose-6-P. Now, with noninhibitory buffers, the activity maximum was broader, extending from *pH* 7.5 to about *pH* 5.9. This result indicated that, in the absence of glucose-6-P, the enzyme could be quite active at the physiological *pH* of yeast (8). It also showed that maleate must be more inhibitory at *pH* 6 than at 7.5, to explain the difference in shape of the two *pH* activity curves. Table 3 shows this as well as that other substances, such as citrate and phosphoenolpyruvate (but not chloride), exhibited the same behavior. In particular, 10^{-2} M phosphoenolpyruvate was almost as inhibitory as a 20 times higher concentration of chloride. Even better inhibitors, at *pH* 5.9, were found in a survey of several nucleotides. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), and guanosine triphosphate (GTP) were efficient at concentrations lower than those of chloride by two orders of magnitude, and glucose-6-P had a large reactivating effect on them. Adenosine monophosphate (AMP), guanosine monophos-

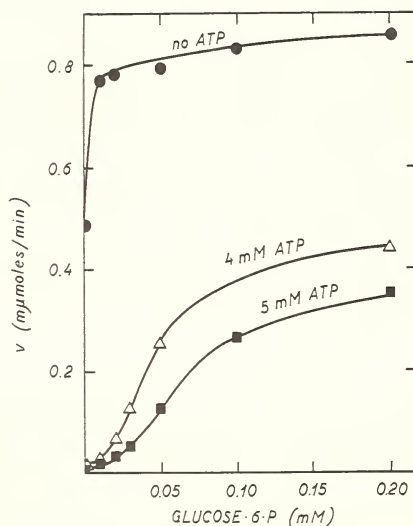
TABLE 3.—Effect of *pH* on enzyme inhibition

Inhibitor	Concentration (mm)	Enzyme activity					
		<i>pH</i> 7.5			<i>pH</i> 5.9		
		–G-6-P	+G-6-P	$\frac{+G-6-P}{-G-6-P}$	–G-6-P	+G-6-P	$\frac{+G-6-P}{-G-6-P}$
None.....	—	100	173	1.7	100	145	1.45
KCl.....	200	40	160	4	46	126	2.7
Maleate.....	100	50	—	—	10.5	—	—
Citrate.....	100	87	—	—	5.5	83	15.1
Phosphoenolpyruvate....	10	86	—	—	55	146	2.7
ATP.....	5	61	146	2.4	10	95	9.5
	1				60	142*	2.4
ADP.....	5	48	160	3.3	14	90	6.4
GTP.....	5	89	178	2	13	90	6.9
UDP.....	0.5	32	72	2.25	42	59	1.4

*With 0.1 mm glucose-6-phosphate (G-6-P).

phate (GMP), and guanosine diphosphate (GDP) were relatively inefficient as inhibitors. The uridine phosphates seemed to belong to the competitive type, as judged by the inability of glucose-6-P to reverse their effect.

Experiments were carried out to determine whether ATP inhibits by the same mechanism as chloride. Text-figure 3 shows that plots of activity against glucose-6-P concentration in the presence of ATP give sigmoid curves, as previously found with chloride (*see* text-fig. 2). Moreover, upon dinitrophenylation, the enzyme lost its sensitivity to chloride and to ATP in parallel fashion. The results were not as clear-cut as those of table 2, because at pH 5.9 the dinitrophenylated enzyme showed some residual sensitivity to the inhibitors.



TEXT-FIGURE 3.—Dependence of reaction rate on glucose-6-phosphate concentration at different levels of adenosine triphosphate. Conditions as in table 3, with succinate-cacodylate buffer at pH 5.9.

In the following discussion, only UDP-glucose will be considered as substrate, and it will be assumed that glycogen is saturating the enzyme at all times. Different lines of evidence indicate that the inhibition found with several anions is of the allosteric type. In the first place, no correlation seems to exist between the effectiveness of the different substances and their structural resemblance to UDP-glucose. Furthermore, the fact that glucose-6-P reverses the inhibition, while it is unable to do so with a true competitive inhibitor such as UDP, supports the hypothesis that the inhibitors do not bind to the substrate site. Final proof for the existence of two different sites comes from the dinitrophenylation experiments, where the sensitivity of the enzyme to the inhibitor was lost, while a large proportion of the original activity was recovered. The fact that the inhibitory power of UDP is

unchanged by dinitrophenylation underlines the difference between competitive and allosteric inhibitors, and indicates an essentially intact substrate site in the dinitrophenylated enzyme.

No cooperative effects of UDP-glucose were found, either in the absence or in the presence of an inhibitor, as shown in text-figure 1. On the other hand, glucose-6-P in the presence of chloride or ATP, and chloride in the presence of glucose-6-P, gave rise to sigmoid curves. These results indicate that both the activator and the inhibitor bind to more than one site of the enzyme. Hill plots (5) of the results obtained when glucose-6-P was varied, gave n values between 1.4 and 2.3. In the case of chloride, n values from 1.3 to 3.1 were obtained. Whether the activator and inhibitor compete for the same sites is not yet clear, but the incomplete reversion by glucose-6-P at sufficiently high inhibitor concentrations militates against this possibility.

The stimulation caused by glucose-6-P in the absence of inhibitors seems to be an independent effect. In the first place, only the maximum velocity of the reaction is modified in this case, whereas in the inhibition by anions the affinity of UDP-glucose for the enzyme is also changed. Furthermore, the activation effect is unspecific, while the reversal of inhibition has been so far obtained with glucose-6-P and glucosamine-6-P only.⁷ It is quite possible that the enzyme possesses a special site or sites for the binding of activating anions.

When the results of this study are considered in terms of the Monod-Wyman-Changeux theory (6), a discrepancy is immediately apparent. According to that theory, the allosteric activators and inhibitors would modify the enzymatic activity by shifting the equilibrium toward one or the other of two configurations. Since the K_m of UDP-glucose changes upon addition of the effectors, its affinity for the two enzymatic forms would be different. Accordingly, a cooperative homotropic effect of UDP-glucose would be expected, at least in the presence of an inhibitor. This effect, however, was not found. An explanation for this disagreement would be that the enzyme possesses several sites for the inhibitor and the activator, but only one site for the substrate. Thus, only inhibitor and activator would exhibit cooperative effects. The presence in an enzyme of different numbers of regulating and catalytic subunits has already been reported (9).

This hypothesis would also apply to the Atkinson-Koshland model (10) if the further restriction were made that the substrate can only bind to the free enzyme.

The results in table 3 show that pH exerts a strong influence on the binding of some inhibitors. Since chloride, which is completely disassociated at both pH 7.5 and 6, shows practically no change in its inhibitory activity, it may be assumed, as a first approximation, that

⁷ Rothman, L. B., and Cabib, E., unpublished results.

these effects are more related to the ionization of the inhibitor than to that of the enzyme.

The *pH* of the yeast cell is probably around 6 (8). The results obtained at this *pH* may therefore be especially significant from the physiological point of view. It is remarkable that under these conditions, ATP inhibits at concentrations not far removed from those found *in vivo* (11, 12). ADP and GTP are also very good inhibitors. At this *pH*, glucose-6-P shows maximal reactivation at a very low concentration, about 0.2 mM.

These effects may play an important role in the regulation of glycogen synthetase *in vivo*. Since both ATP and ADP inhibit, their interconversion is probably of small consequence for the activity of the enzyme. The combined concentration of the two nucleotides may be always large enough to keep the enzyme completely inhibited in the absence of glucose-6-P. The variations of the latter would then determine the level of the enzymatic activity. The concentration of glucose-6-P is probably regulated *in vivo* by the rate of the phosphofructokinase step. This depends, in yeast, on the ATP: AMP ratio (13). When this ratio is high, phosphofructokinase will be inhibited and glucose-6-P will accumulate, thus relieving the inhibition of glycogen synthetase and leading to formation of the polysaccharide. The opposite effect will occur when the ratio decreases.

Aside from this type of control, which is related to the energy state of the cell, there may be another, more connected with growth. Yeast accumulates glycogen when offered large amounts of glucose, in the absence of nitrogen, while addition of ammonium salts reduces the rate of synthesis of the polysaccharide (14). As pointed out by Trevelyan and Harrison (14), this may also occur through the activation of phosphofructokinase and the consequent depletion of glucose-6-P. The carbon of glucose would then be used preferentially to synthesize nitrogenous substances essential for growth, rather than reserve polysaccharides. Before this hypothesis can be accepted, it must be substantiated with measurements of the nucleotides and glucose-6-P levels in yeast, under conditions leading to widely different rates of glycogen synthesis.

RESUMEN

La glucógeno sintetasa de levadura es activada por el glucosa-6-fosfato. Se ha encontrado que varias sustancias aniónicas (cloruro, maleato, etc.) inhiben la enzima en ausencia de glucosa-6-fosfato; la adición de este último restituye la actividad original.

Usando cloruro como efector modelo, se encontró que la inhibición es parcialmente competitiva respecto del UDP-glucosa. Gráficos de la actividad en función de la concentración de UDP-glucosa son hiperbólicos en ausencia o presencia de glucosa-6-fosfato o cloruro. Cuando la actividad se representa en función de la concentración del glucosa-6-fosfato, en presencia de cloruro, se obtiene curvas sig-

moides. Este fenómeno se repite cuando se varía la concentración de cloruro, en presencia de glucosa-6-fosfato. El tratamiento de la enzima con 2-4 dinitrofluorobenceno a pH 8 produce pérdida de la sensibilidad al cloruro, sin mucha pérdida de la actividad. Se concluye que los inhibidores se unen a un sitio diferente del sitio catalítico.

Un estudio de la inhibición a diferentes pH demostró que algunas sustancias inhiben más a pH 6, probablemente el valor fisiológico para la levadura, que a pH 7.5. Los mejores inhibidores son el ATP, ADP y GTP. El glucosa-6-fosfato revierte la inhibición por nucleótidos.

Se propone un mecanismo de control de la síntesis de glucógeno "*in vivo*", de acuerdo al cual, los niveles de glucosa-6-fosfato determinados por la fosfofructoquinasa regulada por los niveles de ATP y AMP, determinarían la velocidad de síntesis de glucógeno.

Existiría un mecanismo de control similar en presencia de iones amonio que canalizaría el carbono de la glucosa hacia la formación de compuestos nitrogenados necesarios para el crecimiento.

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Regulation of Glucose-Phosphorylating Enzymes^{1,2}

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SUMMARY

Kinetic studies of the induction and repression of glucokinase are reported. Glucose phosphorylation is accomplished in the liver of rats and animals of other species by four isoenzymes. One of these, called isoenzyme D or glucokinase, represents about 85% of the total phosphorylating activity and is the most important from the point of view of the regulation of metabolism. Due to its high K_m for glucose (10–20 mM) the enzyme activity is prone to be influenced markedly by the concentrations of the substrate in the liver cells, which may result from changes in the supply of dietary carbohydrate. In addition, the amount of the enzyme protein in the liver depends on the supply of glucose in the diet. In our laboratory when we deprived rats of food or fed a carbohydrate-free diet for several days, this isoenzyme decreased or disappeared. If glucose was given to the animals under these dietary conditions, an abrupt increase in enzyme activity was observed, so that normal values were reached after 8–9 hours of treatment.

This increase is referred to as enzyme induction since it requires *de novo* biosynthesis of protein. Liver glucokinase levels also depend on the presence of insulin. Diabetic rats exhibited very low levels or absence of glucokinase, which was rapidly restored by the simultaneous administration of insulin and glucose. The response to glucose of normal animals fed a carbohydrate-free diet was drastically reduced if anti-insulin guinea-pig serum was given at the same time as glucose. Glucokinase induction may have been abolished when we administered glucagon. Glucocorticoids and growth hormone appeared to regulate the rate of induction but not the final levels of glucokinase activity attained after glucose was given. From results of our experiments with inhibitors of induction, we postulate that glucagon acts as a repressor and glucose as an inducer, the latter requiring the presence of insulin to be effective.—Nat Cancer Inst Monogr 27: 29–40, 1967.

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. O. Cori, p. 41.

DIET AND ADENOSINE TRIPHOSPHATE: HEXOSE PHOSPHOTRANSFERASE ISOENZYMES

It has been reported from our laboratory, as well as from others, that the activity of adenosine triphosphate: D-hexose 6-phosphotransferase from rat liver decreases markedly after the animals have been deprived of food (1-10) or have been fed a carbohydrate-free diet (11-14), and that normal levels are recovered following the administration of carbohydrate for several hours (3, 4, 6-10, 12-15). Changes of activity mainly result from modifications in the activity of one of four isoenzymes of adenosine triphosphate: hexose phosphotransferase. This isoenzyme is called glucokinase (5) (text-fig. 1) and is characterized by its high Michaelis constant for glucose (15). This situation explains the residual activity always observed under different conditions of decreased glucose phosphorylating activity.

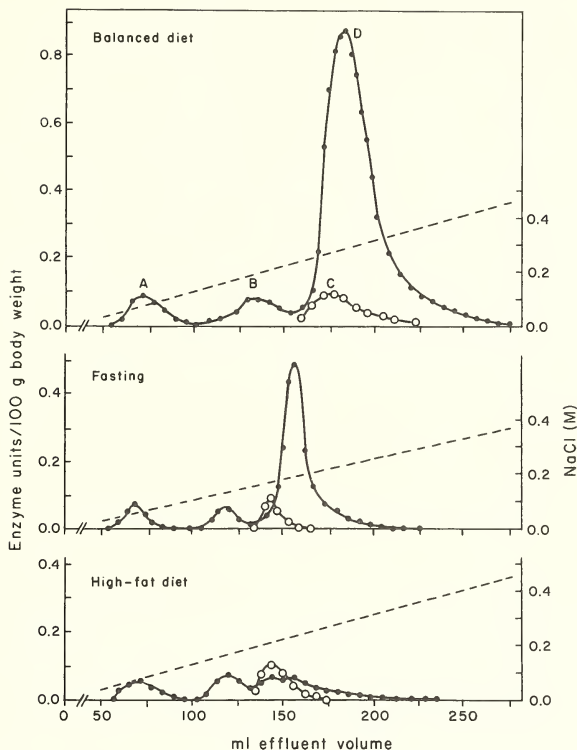
The adaptability of only one of several isoenzymes seems rather common in mammalian tissues, and one can envisage its physiological significance, since it would always permit a certain level of enzyme activity to satisfy the basic requirements of the tissue. The use of substances known to interfere at different stages of protein biosynthesis (4, 6, 7, 10, 12, 14) has supported the hypothesis that the increase in activity after administration of carbohydrate is similar to enzyme induction as it has been described in bacteria. The term "induction" is commonly used to refer to changes in enzyme activities in mammals associated with protein biosynthesis, although there is no evidence in most cases as to whether the changes in enzyme levels reflect a modification in the rate of synthesis or in the rate of degradation, or in both. In some instances these alternatives have been thoroughly studied (16, 17).

KINETIC STUDIES

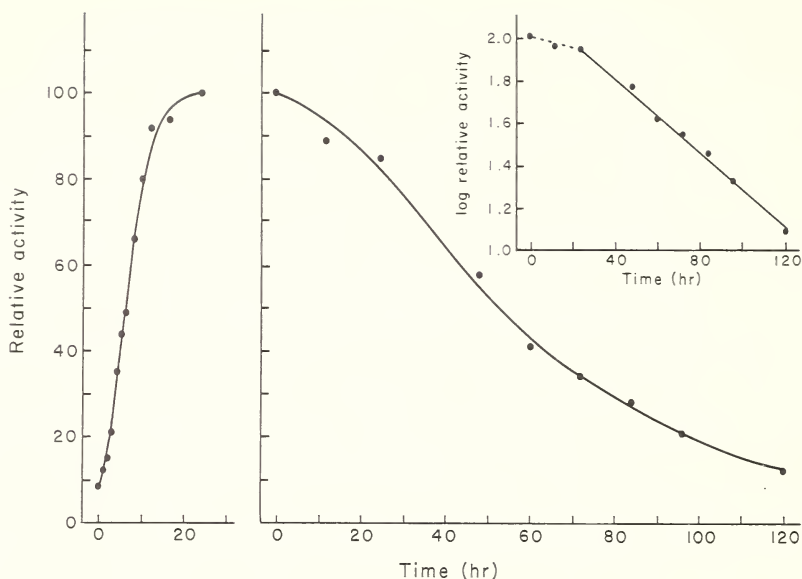
The kinetics of induction and repression of glucokinase differs from that of other enzymes in mammals since the increase in activity is a rapid process, normal levels being recovered after 8-9 hours' administration of carbohydrate following a carbohydrate-free diet (12). On other hand, the decrease of glucokinase occurs slowly, with a half-life of 33 hours (18). These features are illustrated in text-figure 2.

PARTICIPATION OF HORMONES IN GLUCOKINASE CHANGES

In contrast to unicellular organisms, mammals, as well as other multicellular organisms, have an endocrine system that is activated or



TEXT-FIGURE 1.—Chromatographic fractionation of adenosine triphosphate:hexose phosphotransferase isoenzymes from livers of rats under different dietary conditions. Fifty percent (w/v) homogenates were prepared from the pooled livers of several rats in 0.01 M Tris–0.001 M EDTA, pH 7.0 and centrifuged at $105,000 \times g$ in a Spinco preparative ultracentrifuge. The supernatant fluids were treated with CM-cellulose at pH 7.0 and then chromatographed on a DEAE-cellulose column. Linear gradient elution was carried out with NaCl from 0–0.5 M (— — —) in 0.01 M Tris–0.001 M EDTA, pH 7.0. Fractions of 3 ml were collected, and the content of each tube was examined for protein and for phosphorylating activity. ATP:hexose phosphotransferase activity was measured following the reduction of NADP in a Beckman DU spectrophotometer in the presence of ATP, Mg ions, and an excess of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (8). The enzyme activities were assayed at 100 mM (●), and 0.5 mM (○) glucose concentrations. The same systems with ATP omitted acted as blanks. One unit of ATP:hexose phosphotransferase corresponds to the amount of enzyme that phosphorylates 1 μ mole of glucose in 1 minute at 30 C. Data from González *et al.* (15).



TEXT-FIGURE 2.—Kinetics of glucokinase induction and repression. To study the *enzyme induction*, several rats (strain A \times C) were fed a high-fat carbohydrate-free diet for 6 days. On the 7th day they received a carbohydrate solution (20% glucose-30% dextrin) by stomach tube, and this treatment was repeated when it corresponds every 4 hours. Groups of 4-5 animals were killed at the time intervals indicated in text-figure 2 and the livers removed for the assay of glucokinase, which is approximately estimated by the difference in activity measured at 100 mM and 0.5 mM glucose (6-8). To study *glucokinase decay*, animals fed with a balanced diet were transferred to a high-fat carbohydrate-free diet and killed at varying time intervals for liver glucokinase assay. Data from H. Niemeyer, J. Babul, G. Chamorro, and R. Schilkrut (18).

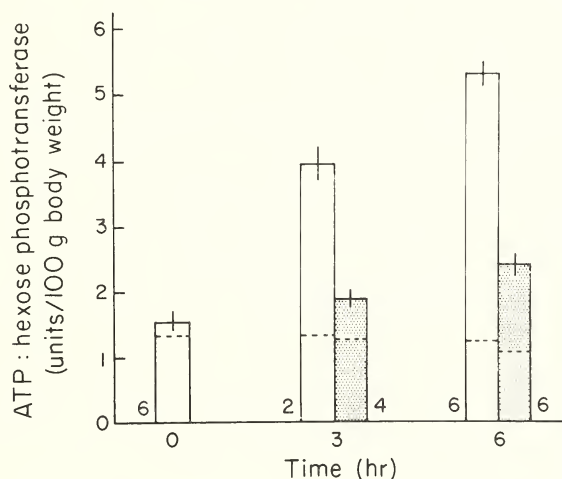
inhibited through several mechanisms and contributes to the regulation of metabolism in tissues. The amount and nature of the diet may stimulate or depress selectively the secretion of different glands, making it difficult to discriminate which effects on tissue metabolism and enzyme patterns obtained by modifications of the diet are the result of changes in endocrine activities and which are the direct consequence of the components of the diet (2, 19).

Insulin

In alloxan-diabetic rats, there has been observed a marked decrease of phosphotransferase activity at the expense of glucokinase, the high K_m isoenzyme (1, 5-7, 20-23). The administration of insulin restores normal activity (6, 7, 20-23). Insulin is also essential for the induction of ATP:hexose phosphotransferase in diabetic rats deprived of

food (20) or fed a carbohydrate-free diet (22). On the other hand, the injection of insulin into normal rats fed a carbohydrate-free diet does not promote the induction of glucokinase, with the use of different doses and different schedules of administration (19).

Most experiments that point out the necessity of insulin, either for the maintenance of normal levels of enzyme activity or for glucokinase induction after enzyme depletion, have been performed with diabetic rats which are in a rather chronic condition for at least several days or weeks. The administration of *anti-insulin serum* from guinea pigs to animals of several species results in an acute insulin insufficiency shown by the occurrence of hyperglycemia and occasionally glucosuria and ketonuria (24-27). Such a procedure permits the study of effects of insulin deprivation, with minimal interference of secondary modifi-



TEXT-FIGURE 3.—Effect of insulin anti-serum on the substrate induction of glucokinase in rat liver. Rats previously fed a high-fat carbohydrate-free diet for 6 days were submitted to various treatments before being killed for enzyme assay. Ten rats received intravenously 0.5–0.7 ml of anti-insulin guinea-pig serum and 15 minutes later a solution of carbohydrate by stomach tube. Four of these animals were killed 3 hours after administration of carbohydrate. The other 6 received a second dose of immune serum and carbohydrate at this time and were killed 3 hours later. Groups of rats similarly treated in which normal guinea-pig serum replaced the immune serum acted as controls. A group of 6 animals did not receive any treatment and acted as controls (time 0). Bars represent total phosphorylating activity measured with 100 mM glucose and dotted lines indicate parallel assays with 0.5 mM glucose (approximate estimation of low- K_m isoenzymes). Vertical lines at top of bars indicate standard deviation, with the exception of the clear bar at 3 hours where it indicates the range of activities. Dotted bars correspond to animals treated with anti-insulin serum and clear bars to control rats. Figures beside bars indicate the number of rats in each group. Data from H. Niemeyer, N. Pérez, and R. Codocco (22).

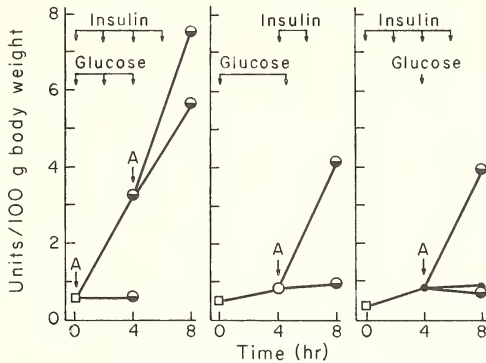
cations caused by long-standing insulin deprivation. On the other hand, it overcomes the interference of secondary effects of the surgical procedure when rats are to be used a few hours after pancreatectomy. While removal of insulin by antiserum may abolish synthesis of glucokinase, this effect cannot be readily discerned by measurements of the steady-state level of the enzyme because of its apparent low turnover. To circumvent this difficulty we have studied the effect of anti-insulin serum on enzyme induction (22).

Text-figure 3 shows that the induction of glucokinase by glucose in normal rats previously fed a carbohydrate-free diet is markedly depressed by the intravenous injection of anti-insulin guinea-pig serum. The concentration of glucose in blood was higher in the animals receiving the antiserum than in the controls injected with normal guinea-pig serum and deposition of glycogen was prevented by the immune serum. The administration of insulin at the same time as the immune serum antagonized the effect of the latter (22).

It has been reported that administration of insulin to alloxan-diabetic rats permits a *slow* recovery of liver glucokinase activity after a lag phase of several hours (6, 7, 23). However, the kinetics of the enzyme induction shown in text-figure 4 is quite different. These results are for diabetic rats submitted to slightly different experimental conditions, since the animals were fed a carbohydrate-free diet for several days (22). It is shown that the *administration of insulin at the same time as carbohydrate* promotes the rapid increase in glucokinase activity, while neither glucose nor insulin alone is effective. The low levels of enzyme activity observed in the nontreated animals or in the rats that did not respond with an increase in phosphorylating activity after treatments could be accounted for by the hexokinase-type isoenzymes. In kinetic experiments of this type it has been observed that the response of liver depends on the dose of insulin administered in relation to the severity of the diabetic condition (22).

The observations on animals treated with anti-insulin serum, as well as the experiments in which the simultaneous provision of carbohydrate and insulin induced liver glucokinase in diabetic animals, indicate that both insulin and glucose must be presumptively present at the same time in the liver and at appropriate concentrations in order to start the synthesis of the enzyme protein.

The kinetics of glucokinase induction in diabetic animals shown here differs from that given in data from other laboratories (6, 7, 20, 23). In these laboratories, a lag phase followed by a slow increase in glucokinase after administration of insulin has been observed. Perhaps the difference is due to the fact that in our experiments there was an overload of glucose simultaneous with the administration of insulin. The severity of the diabetic state, which is difficult to evaluate prop-



TEXT-FIGURE 4.—Effect of separate or simultaneous treatment with carbohydrate and insulin on glucokinase induction in liver of diabetic rats. Groups of rats were fed with a balanced diet for 4 days after injection of 200 mg alloxan-monohydrate per kg body weight by the intraperitoneal route, and then fed with a high-fat carbohydrate-free diet for 6 days. In each experiment, one group was killed as initial control and others were treated with carbohydrate (glucose and maltose-dextrin) and/or insulin at the times indicated by the arrows. Several animals were injected intraperitoneally with 100 μ g of actinomycin D at times indicated by arrow A. The animals were killed at varying periods after treatment began. Each point represents the mean of total phosphorylating activity in groups of 4–6 rats. □ = controls without glucose and insulin; ○ = rats treated only with carbohydrate. ● = animals treated only with insulin; ● = animals that received both insulin and carbohydrate. Data from H. Niemeyer, N. Pérez, and R. Codoceo (22), and from H. Niemeyer, N. Pérez, and E. Rabajille (28).

erly, may be another variable determining the different response. It must be emphasized that feeding the diabetic rats a carbohydrate-free diet (the routine procedure in the experiments presented here) alleviates the condition of the animals; the level of blood glucose is generally lower than in animals fed a balanced diet, glucosuria and loss in weight are proportionally less accentuated, and survival is higher. However, ketonuria is permanent.

As shown in text-figure 4, actinomycin prevents glucokinase induction and its effect depends on the time of administration during enzyme induction. If given at the start of the process, at the same time as the first dose of carbohydrate, there is no response at all. On the other hand, if the drug is administered after several hours of enzyme induction, the increase of enzyme level continues at a slightly lower rate for several hours. The different behavior of the system is interpreted as the result of a block by actinomycin of the transcription from DNA to messenger RNA. The messenger formed during the 4 hours of induction would permit the biosynthesis of the phosphotransferase to continue for a certain period of time, which depends on the degradation rate of the mRNA. This interpretation has been used in

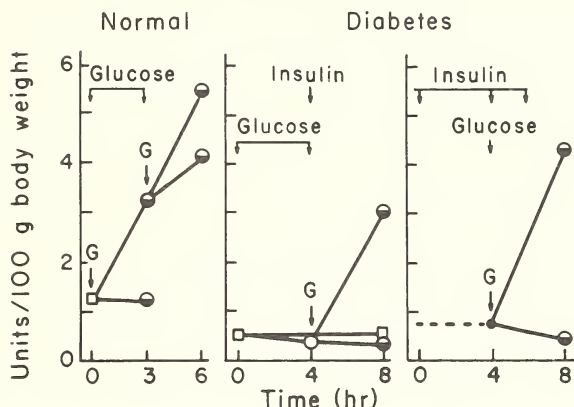
an attempt to elucidate the role of insulin and that of glucose in glucokinase induction.

One possibility is that glucose acts as the inducer, *i.e.*, it is the agent responsible for the formation of the specific mRNA for glucokinase, and that insulin facilitates some subsequent step in the process of biosynthesis. Another possibility is that insulin is the inducer and that the substrate acts to stabilize the newly formed enzyme. The injection of actinomycin after 4 hours of separate action of glucose (text-fig. 4, *center*) or insulin (text-fig. 4, *right*) blocks completely the induction of glucokinase. This indicates that during the interval there was no accumulation of messenger RNA which could be active in the second period when both agents, insulin and carbohydrate, are present. The conclusion from these experiments is that the simultaneous presence of glucose and insulin is required for the transcription from DNA during glucokinase induction.

Glucagon

It has been briefly reported that glucagon and epinephrine prevent the increase of ATP:hexose phosphotransferase activity that occurs after feeding animals previously deprived of food for several days (10). We have confirmed and expanded these observations in animals previously fed a carbohydrate-free diet (19). Glucagon prevented the induction of phosphotransferase by carbohydrate, the effect being clearly dependent on the dose. With as small a dose as 1 μ g every 75 minutes, an increase in enzyme activity slightly less than that in the controls without glucagon was observed. However, a significant glycogenolytic effect was evident with that dose. Administration of 100 μ g every 3 hours completely abolished phosphotransferase induction in normal rats fed a carbohydrate-free diet. Even the highest doses of glucagon used (300 μ g every 3 hours) did not modify the enzyme activities in livers from rats fed a carbohydrate-free or a balanced diet during a 6-hour period. Glucagon was equally effective in preventing enzyme induction in the presence and absence of exogenous insulin. The effect of glucagon depended on the time of injection in relation to the administration of carbohydrate, as illustrated in text-figure 5. If glucagon was given 3 hours after carbohydrate, while enzyme activity was increasing, it inhibited but did not block the enzyme increase. The effect of glucagon was therefore like that of actinomycin.

Glucagon was given to diabetic rats in experiments similar to those previously described in which actinomycin was administered. When given to alloxan-diabetic rats fed a carbohydrate-free diet after the administration of either glucose alone (text-fig. 5, *center*), or insulin alone (text-fig. 5, *right*), glucagon prevented completely the increase in glucokinase that follows the supply of the complementary agent,



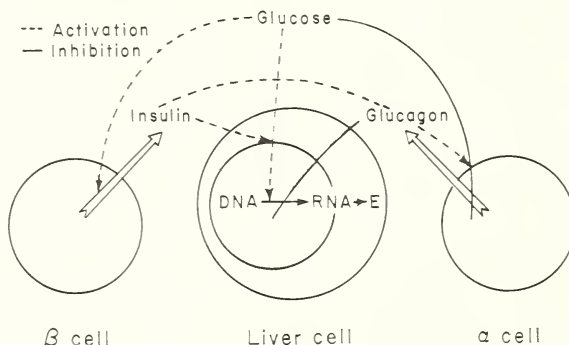
TEXT-FIGURE 5.—Influence of glucagon administration on the induction of ATP:hexose phosphotransferase in normal and diabetic rats. The design of the experiments and the symbols are the same as in text-figure 4, except that glucagon was given at arrows G. Data from H. Niemeyer, N. Pérez, and E. Rabajille (19, 28).

insulin (*center*) or glucose (*right*), respectively. As in the case of actinomycin, these experiments are considered as an indication of the necessity of the simultaneous presence of glucose and insulin to produce a specific messenger RNA for glucokinase.

From these results it might be suggested that glucagon is a physiological repressor and that glucose can act as an inducer in the liver cell. The relative levels of glucagon and glucose would be the signal to switch *on* or to switch *off* the biosynthesis of phosphotransferase. Because of the similarity, in many respects, between total fasting and a high-fat carbohydrate-free diet, it is quite possible that animals on the latter regimen present, as fasted animals do (29), a higher than normal level of blood glucagon which, together with a relatively low glucose concentration in the liver cell, would be responsible for the low activity of liver ATP:hexose phosphotransferase.

What speculations can we make with respect to the function of insulin? It is accepted that the external membrane of the liver cell is not affected by insulin and is thus freely permeable to glucose (30–32). One can consider the possibility that insulin may act upon internal membranes of the cell. It could be that insulin is responsible for the functional integrity of the endoplasmic reticulum, which would be in line with the well-recognized role of insulin in protein biosynthesis (33, 34). Another possibility, which would give more specificity to insulin action, is that glucose entry into the nucleus in the liver cell is insulin-dependent. The permissive role of insulin would be to facilitate the action of glucose, the true inducer, at the DNA level.

Text-figure 6 presents schematically the possible roles of glucose, glucagon, and insulin on glucokinase induction in the liver. Alternative explanations for insulin action might be discussed, but we do not have time for further speculations here.



TEXT-FIGURE 6.—Schematic representation of the hypothetic role of glucose, glucagon, and insulin on glucokinase induction in liver cells. *Glucose* might be responsible for several effects: 1) secretion of insulin, 2) blockage of glucagon secretion, and 3) derepression of glucokinase-mRNA formation. *Glucagon* would be a physiological repressor for glucokinase. *Insulin* would have a permissive effect acting at the nuclear membrane, facilitating the action of glucose; it could also act at the membrane of the α -cell, permitting glucose to block glucagon secretion.

RESUMEN

En el hígado de ratas y de otras especies la fosforilación de la glucosa es catalizada por medio de cuatro isoenzimas. Una de estas, llamada isoenzima D o glucoquinasa, representa alrededor de un 85% de la actividad fosforilante total y es la más importante desde el punto de vista de la regulación del metabolismo. Debido a su alta K_m para glucosa (10–20 mM), la actividad enzimática puede ser influenciada en forma acentuada por las concentraciones del sustrato en la célula hepática que resulten de cambios en el aporte hidrocarbonado en la dieta. Además, la cantidad de proteína-enzima en el hígado depende del aporte de glucosa en la dieta. Así, esta isoenzima disminuye o desaparece cuando las ratas son sometidas a ayuno o son alimentadas con una dieta exenta de glúcidos durante varios días. Si se suministra glucosa después que los animales hayan estado bajo estas condiciones, se observa un brusco aumento de la actividad enzimática, alcanzándose los valores normales después de 8 a 9 horas de tratamiento. Este aumento se considera como inducción enzimática pues requiere biosíntesis *de novo* de proteína. Los niveles de glucoquinasa también dependen de la presencia de insulina. En las ratas diabéticas se encuentran niveles muy bajos o ausencia de glucoquinasa. La enzima recupera los valores normales rápidamente después de la administración simultánea de insulina y glucosa. La respuesta a la glucosa de los animales normales alimentados con una dieta exenta de hidratos de carbono es reducida drásticamente si se inyecta suero anti-insulina preparado en cuy, al mismo tiempo que se suministra

glucosa. La inducción de glucoquinasa puede también ser abolida por glucagón. Los glucocorticoides y la hormona de crecimiento parece que regulan la velocidad de la inducción pero no los niveles finales de actividad glucoquinásica, obtenidos después de la administración de glucosa. Experimentos realizados con inhibidores de la inducción permiten postular que el glucagón actúa como un represor y la glucosa como un inductor, y que la presencia de insulina es necesaria para que la glucosa sea efectiva.

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DISCUSSION ¹

Discussor, DR. OSVALDO CORI, Department of General Biochemistry, University of Chile, Santiago, Chile

AN EARLIER session offered us, besides Dr. Leloir's panoramic view of glycogen metabolism, examples of two main aspects of regulatory mechanisms: *a*) The effect of allosteric ligands on the enzyme molecule, and *b*) the superimposed hormonal mechanisms controlling, among other things, the biosynthesis of enzymes. I have always been intrigued by the contrast existing between the similarity of metabolic pathways in widely differing organisms, on one hand, and the lack of uniformity in regulatory mechanisms on the other. I wish to call your attention to some of the differences between regulatory mechanisms in various organisms.

In contrast to muscle phosphorylase, potato phosphorylase ² is not controlled by the adenylic acid system. It cannot be phosphorylated by ATP + phosphorylase *b* kinase, thus probably excluding any glucose-1-phosphate/glucose-6-phosphate regulatory mechanism as demonstrated for the muscle enzyme by Dr. E. Fischer. A recent report by Frydman and Cardini ³ on potato ADPG-starch glycosyltransferase does not mention any regulation of this enzyme by glucose-6-phosphate as in yeast and liver.

In the absence of an allosteric control of the enzymes involved in starch biosynthesis and breakdown, one may wonder how the process leading to starch accumulation in the vegetating potato tuber is reversed when the young plant begins to grow.

It is conceivable that the allosteric regulation of polysaccharide synthesis may still take place at an earlier step. Preiss ⁴ has described such an effect in spinach chloroplast: 3 phosphoglyceric acid, an early photosynthetic product, is a "sigmoid" stimulant of ADPG pyrophosphoryl-

¹ Of articles by Luis F. Leloir; Enrico Cabib and Lucia B. Rothman; and Hermann Niemeyer.

² Brown, D. M., and Cori, C. F.: *In The Enzymes* (Boyer, P. D., Lardy, H. A., and Myrbäck, K., eds.), New York, Academic Press Inc., vol 5, 1961, p 209.

³ Frydman, R. B., and Cardini, C. E.: *Arch Biochem* 116: 9, 1966.

⁴ Gosh, H. P., and Preiss, J.: *J Biol Chem* 241: 4491, 1966.

ase at pH 8.5, whereas ADP and P_i are inhibitors. This would provide an effective regulatory coupling between photosynthesis and starch deposition. A similar stimulation has been described⁵ for the same enzyme from *Arthrobacter viscosus* by fructose-6-phosphate and pyruvate.

Temperature has been shown to affect enzyme activities in vegetating potato tubers in opposing directions.⁶ Cold stored potato tubers contain less phosphohexose isomerase and aldolase activities than controls (23°), while invertase shows an increased activity in phase with the increase in reducing sugar contents.

Another mechanism of increase in enzyme activity during maturation has been observed in germinating wheat and barley. β -Amylase is bound by S-S linkages to storage protein and is released on maturation by reduction of the disulfide bonds.⁷

Thus, it looks as if allosteric regulation may well be the basis for the control of moderately rapid changes, such as those occurring in microorganisms or in the light-dark transition in green leaves. Hormonal trigger and amplification complete the picture in the fast-reacting animal tissues.

It is also true, however, that hormones play a role at the other end of the rate spectrum: Gibberellins accelerate the growth and shorten the dormancy of potato tubers in the field, with concomitant increase in the ability of its chromatin to serve as a template for RNA.⁸

That this may be an enzymic induction (or derepression?) has been well substantiated by Varner.⁹ He has been able to show that 10^{-6} M gibberellic acid induces increased biosynthesis of α -amylase in barley seeds. This increase in enzyme biosynthesis is concomitant with an increased rate of incorporation of bases and of ^{32}P into RNA. It is perhaps not too risky to speculate that similar mechanisms may be operating in the change from vegetating to active plant tissues, and this might be a fruitful area of exploration.

I hope that this little digression may encourage other participants to complement the discussion with evidence from the heretofore somehow neglected realm of plants.

In the open discussion following Dr. Cori's remarks, Dr. Changeux inquired whether binding studies had been done with glycogen syn-

⁵ Shen, L., and Preiss, J.: Arch Biochem 116: 375, 1966.

⁶ Tishel, M., and Mazelis, M.: Phytochem 5: 895, 1966.

⁷ Roswell, E. V., and Goad, L. J.: Biochem J 84: 73P, 1962.

⁸ Bonner, J.: In Plant Biochemistry (Bonner, J., and Varner, J. E., eds.). New York, Academic Press Inc., 1965, p 859.

⁹ Varner, J. E., Chandra, G. R., and Chrispeels, M. J.: J Cell Comp Physiol 66 (Suppl I): 655, 1965.

thetase and Dr. Cabib indicated that as yet only kinetic evidence was available.

Dr. Fischer asked if the activation of glycogen synthetase by calcium is reversible, and was told by Dr. Leloir that it is not. Fischer noted that Dr. E. G. Krebs and Dr. B. Houston have shown a similar irreversible activation of phosphorylase by calcium and a protein cofactor; in this instance the protein component was found to be a specific proteolytic enzyme. Fischer briefly described recent experiments in his laboratory indicating a second type of activation by calcium which is reversible.

The potential role of adenylate kinase as a regulatory factor in the metabolism of glycogen was discussed by Dr. Gaede, who referred to several indications that the ratios ATP/AMP and ADP/AMP are determining factors for glycogenolysis or glycogen synthesis. Gaede pointed out that these ratios, in turn, are profoundly influenced by adenylate kinase, a rather ubiquitous enzyme that he has recently found in epithelial cells of the intestinal mucosa. He described shifts in the equilibrium constant of the adenylate kinase reactions at various Mg^{++} concentrations that could give marked changes in the intracellular distribution of phosphorylated adenine nucleotides.

Dr. Gaede introduced discussion of Dr. Niemeyer's paper by asking if glucagon acts immediately (within a few minutes) in repressing enzyme synthesis, in accord with previous experiments which emphasized the rapidity of response to glucagon in contrast to the slower response to insulin. Dr. Hans Krebs countered this view, stating that both glucagon and insulin have multiple effects, some immediate and some delayed. Krebs described some recent experiments on glucagon effects in the isolated, perfused liver of the rat. Glucagon acts quickly to stimulate glycogenolysis in the well-fed liver, but promotes gluconeogenesis from lactate and pyruvate in the starved liver. Another illustration of the multiple effects of hormones is the effects of adrenalin on blood pressure and glucose metabolism, as Krebs pointed out.

Dr. Leloir raised the question of how glucose could be an inducer of glucokinase in view of the fact that glucose is always present and in rather constant concentration. Dr. Niemeyer indicated that the glucose content of the portal blood is higher than that in the systemic blood in animals fed carbohydrates; the reverse is true in animals fed a carbohydrate-free diet. He indicated that this variance may be significant in providing a high level of glucose in the liver, which is probably necessary for glucokinase induction.

Dr. Cabib inquired whether the glucose effect, rather than that of insulin, might be "permissive" insofar as it may be necessary to metabolize glucose for some time to remove some resistance to the

action of insulin. Dr. Niemeyer admitted this as a possibility that cannot be ignored, but then gave several points which suggest that the metabolism of glucose may not be important. For example, other sugars (fructose, galactose, mannose), which would yield the same metabolites, are not as effective as glucose in enzyme induction. The view that the glucose may reflect its capacity to stabilize glucokinase would seem to be unlikely since derivatives of glucose (*e.g.*, 2-deoxy-glucose) that stabilize the enzyme *in vitro* do not promote any increase in glucokinase *in vivo* even when given together with insulin.

MONDAY AFTERNOON

Chairman: Mario Garcia-Hernández

The Activation and Inactivation of Muscle Phosphorylase^{1,2,3}

EDMOND H. FISCHER, SUZANNE S. HURD, PEARL KOH, and DAVID TELLER, *Department of Biochemistry, University of Washington, Seattle, Washington 98105*

SUMMARY

The activity of muscle phosphorylase is determined and, at times, controlled by a number of factors. These include phosphorylation of the protein (*i.e.*, conversion of phosphorylase *b* to *a* by phosphorylase kinase), activation by adenosine 5'-phosphate, presence of pyridoxal 5'-phosphate, state of aggregation of the molecule. In all cases, specific regions of the protein molecule are involved, and regulation of enzymatic activity can be visualized as resulting through a number of site-site interactions. Evidence is presented indicating that the activity of the enzyme is controlled by yet another parameter: When phosphorylases *a* and *b* are converted into one another, phosphorylation of the protein does not proceed in an all-or-none reaction as originally postulated, but in a stepwise fashion in which partially phosphorylated intermediates are produced. These "phospho-dephospho" hybrids show catalytic activity and physicochemical be-

havior different from either phosphorylase *b* or *a*: They possess high enzymatic activity when measured (in the direction of glycogen synthesis) at high concentrations of glucose-1-phosphate (G1P) but this activity is greatly depressed by low amounts of glucose-6-phosphate (G6P). Likewise, their sedimentation behavior, as observed in the ultracentrifuge, is strongly affected by the presence of the sugar esters: G1P increases the proportion of the heavy component (presumably corresponding to the tetramer) while G6P favors formation of the dimeric form. A model attempting to explain these phenomena in terms of the molecular structure of the enzyme, and consistent with the data, is presented. Interaction of pure phosphorylases *a* and *b* under various conditions was also studied and evidence obtained that an *a*₂*b*₂ hybrid is produced. The role of these partially phosphorylated intermediates in the regulation of phosphorylase activity is discussed.—*Nat Cancer Inst Monogr* 27: 47-59, 1967.

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. H. A. Lardy, p. 125.

³ This work was supported by Public Health Service grants AM-07902 and GM-13401 from the National Institute of Arthritis and Metabolic Diseases.

⁴ Abbreviations used are as follows: AMP, adenosine 5'-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; pCMB, parachloromercuribenzoate.

THIS REPORT concerns the mechanism of activation of muscle phosphorylase, particularly as it relates to the structure of the enzyme. From the extensive work of Cori and his group (1), we know that phosphorylase exists in two forms: phosphorylase *b*, a dimer of m.w. 250,000, inactive in the absence of AMP, and phosphorylase *a*, a tetramer of m.w. 500,000, active in the absence of this nucleotide. Both phosphorylase *b* and *a*, when treated with an excess of pCMB, dissociate into monomer units of m.w. 125,000: nonphosphorylated units in the case of *b* and phosphorylated monomers in the case of *a* (2). This dissociation can be reversed by addition of excess cysteine. As will be discussed, conversion of phosphorylase *b* to *a* occurs through phosphorylation of the protein by ATP and phosphorylase *b* kinase; phosphorylase *a* is converted back to *b* by a specific phosphorylase phosphatase (3).

One very notable characteristic of phosphorylase is that this protein contains a multiplicity of sites, all involved in some way in determining or controlling the activity of the enzyme. Of course, this is not a feature unique to phosphorylase, but one being encountered more and more often in regulatory enzymes.

First, there is the catalytic site, binding the various substrates and some inhibitors. Nothing is known, we believe, about the nature of the residue on the protein forming this active site. Then, there is a site binding nucleotides; it is the binding of one mole of AMP per mole of monomer which renders phosphorylase *b* enzymatically active. From the work of Parmeggiani and Morgan (4), Madsen (5), and others, it is known that the site also binds ATP since these two nucleotides compete with one another. Thirdly, there is the site phosphorylated during the *b* to *a* conversion; covalent substitution of the protein in some way "freezes" the molecule in the active conformation. We know the amino acid sequence around that seryl residue which is phosphorylated. Some information is also available regarding a fourth site on phosphorylase, *i.e.*, the group involved in the binding of pyridoxal 5'-phosphate; this cofactor, indispensable to the activity of the enzyme, is bound to an ϵ -amino group of a lysyl residue adjacent to a phenylalanyl residue (6). Finally, there must be sites or a distribution of groups involved in the aggregation of the molecule: two at least, since one has to account for the formation of the *b* dimer and the *a* tetramer. Nothing is known, however, about the groups involved in the aggregation of phosphorylase, nor is information available on the general topography of the molecule itself and whether or not some sites might be overlapping.

Many site-site interactions have been recognized that in some way affect the activity of the enzyme. Time, however, will permit discussion only of some properties of the seryl phosphate site, particularly

as they relate to the mechanism of action of the kinase and the phosphatase, and affect the interaction of the phosphorylase subunits. The properties of the pyridoxal phosphate site have been recently summarized (7-9).

SERYL PHOSPHATE SITE

The site phosphorylated during the interconversion of phosphorylase *b* and *a* has the following sequence (10) :



This site is not required for catalytic activity since the removal of a hexapeptide fragment following tryptic attack (between the two arrows) leaves a molecule enzymatically active when assayed in the presence of AMP (10). The phosphorylated tetradecapeptide isolated from phosphorylase is slowly dephosphorylated by phosphorylase phosphatase, and the dephospho peptide obtained, in turn, is extremely slowly rephosphorylated by phosphorylase *b* kinase and ATP. Though this indicates a certain degree of recognition for the primary sequence of the seryl phosphate site on behalf of these two enzymes, clearly, this enzyme is enhanced by at least two orders of magnitude when the native enzyme is used as substrate.

The molecular state of phosphorylase, and hence its activity, is regulated by the opposite actions of phosphorylase kinase and phosphatase (we will neglect for the present the activation of phosphorylase *b* by AMP even though this mechanism of activation is clearly of importance). These two enzymes are part of an elaborate system of control, involving a number of enzymes, nucleotides, divalent metal ions, hormones, etc., ultimately leading to the formation of enzymatically active or inactive molecules. A detailed description of the successive steps leading to the interconversion of phosphorylase *a* and *b* has been summarized elsewhere (3, 11). This paper will be solely concerned with the structural changes affecting the phosphorylase molecule itself, particularly as revealed by a study of the phosphorylase phosphatase reaction.

PHOSPHORYLASE PHOSPHATASE REACTION

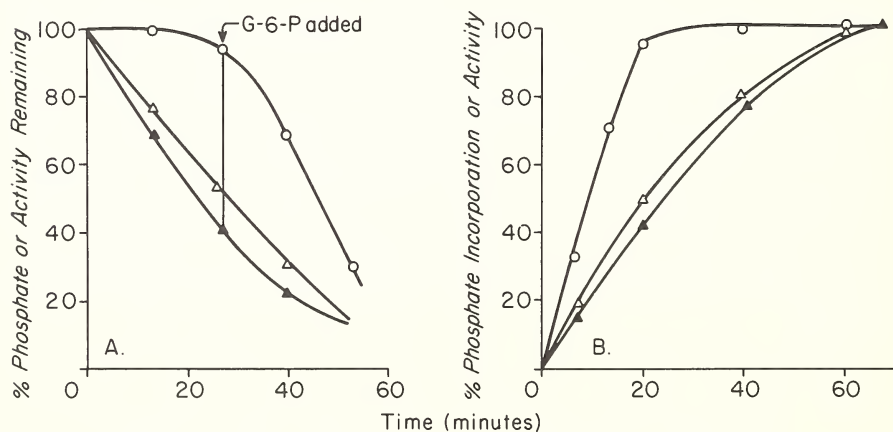
Phosphorylase phosphatase has been purified approximately 300-500-fold from crude rabbit-muscle extract (12). The procedure involves the precipitation of the enzyme as a glycogen complex and its

liberation from this complex by α -amylase followed by successive TEAE-cellulose and Sephadex G-200 chromatographies. The material obtained is far from homogeneous, as judged by acrylamide gel electrophoresis; however, the band representing the phosphatase could be identified enzymatically; apparently, at least another 10-fold purification will be required to yield a homogeneous preparation.

Phosphorylase phosphatase has a molecular weight in the order of 60,000 as evaluated by sucrose density gradient centrifugation. The purified enzyme is sensitive to temperatures above 45 C, but is resistant to tryptic and chymotryptic attack; it will also withstand treatment in 8 M urea for several hours at room temperature (advantage was taken of these properties in attempts to further purify the enzyme). The enzyme has a K_m of 2×10^{-6} for phosphorylase *a* and a turnover of approximately 2.5 moles phosphorylase *a* (m.w. 500,000) dephosphorylated per minute per 60,000 g phosphatase.

A few years ago, when the phosphorylase reaction was studied by release of ^{32}P from ^{32}P -labeled phosphorylase *a* and by loss of phosphorylase *a* activity, it was reported that these two reactions paralleled one another (13). Lately, however, it was found that if the phosphorylase *a* remaining in the reaction was measured at high G1P concentration (≥ 0.05 M), no loss of activity could be observed even though close to 50% of the protein-bound phosphate had been released (text-fig. 1A).

If phosphorylase was measured at the low concentration of G1P used originally (0.016 M), or if as little as 0.002 M G6P was added to the assay system, loss of activity paralleled or even exceeded the loss



TEXT-FIGURE 1.—Effect of G1P on phosphorylase activity during *a* → *b* reaction catalyzed by phosphorylase phosphatase (A) and *b* → *a* reaction catalyzed by phosphorylase kinase (B). Δ , release or incorporation of ^{32}P ; O, phosphorylase activity measured at 0.75 M G1P; \blacktriangle , measured at 0.1 M G1P + 0.001 M G6P.

of ^{32}P . These results were interpreted by assuming that the four phosphate groups bound to phosphorylase *a* were not released simultaneously, in an all-or-none reaction as originally postulated, but in a step-wise fashion, giving rise to partially phosphorylated intermediates having successively 3, 2, 1, and finally zero phosphate groups. It was further assumed that the enzymatic activity of these intermediates (phospho-dephospho hybrids) would be greatly affected by the concentration and relative proportion of the sugar esters: They would be enzymatically active when measured at high G1P concentration, but predominantly inactive in the presence of G6P. The activity of pure phosphorylase *a* is totally unaffected by the presence of G6P.

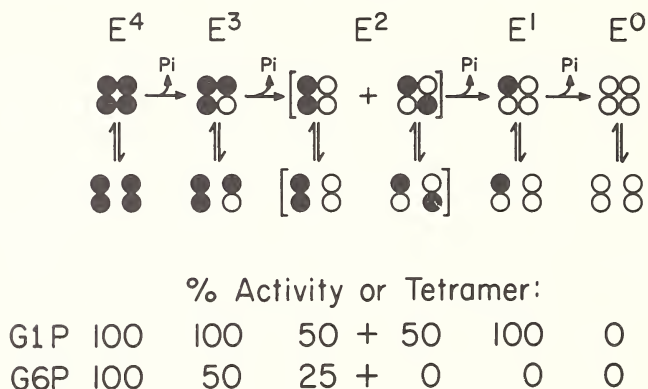
The above hypothesis implies that the occurrence of these phospho-dephospho hybrids is a unique characteristic of the phosphorylase molecule itself, not of the phosphatase. This is shown by the fact that the same partially phosphorylated intermediates are produced in the reversed reaction, namely the conversion of phosphorylase *b* \rightarrow *a* catalyzed by phosphorylase *b* kinase (text-fig. 1*B*).

A MODEL FOR THE PHOSPHATASE REACTION AND THE PRODUCTION OF PHOSPHO-DEPHOSPHO HYBRIDS

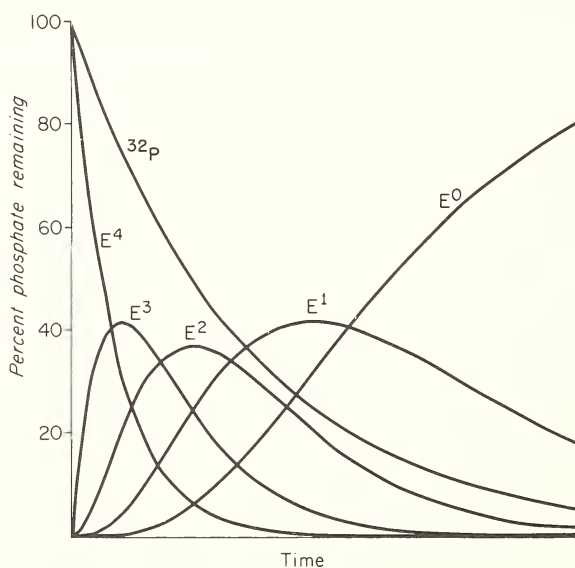
To investigate the kinetics of the phosphatase reaction, the following model was proposed as a working hypothesis: *a*) in the phosphatase reaction, phosphorylase *a* exists as a tetramer containing one bound phosphate group per monomer unit; *b*) each of these four phosphate groups is hydrolyzed at the same rate (even though there are indications that phosphorylated species existing in the dimeric form are more susceptible to the action of the phosphatase); *c*) each intermediate species produced in the reaction is in a state of equilibrium between two conformations, resulting in the formation of either tetramers or dimers. For fully phosphorylated species the equilibrium strongly favors tetramer formation, while totally dephosphorylated species, *i.e.*, phosphorylase *b*, dissociate into dimeric units; and finally, *d*) this equilibrium is affected by a number of factors: G1P or AMP promote tetramerization while G6P or glucose favors the dimeric species. The following scheme was therefore proposed (text-fig. 2).

The formation of each intermediate was computed on an analogue computer, as illustrated in text-figure 3.

From text-figure 3, the percentage of each intermediate species for any amount of phosphate released can be calculated (*see* table 1). According to points *c*) and *d*) listed above, partially phosphorylated species could have full enzymatic activity when measured at high G1P concentration, while they would be totally inactive if G6P is present.

Model for Phosphorylase $a \rightarrow b$ Reaction

TEXT-FIGURE 2.—Hypothetical scheme for conversion of phosphorylase $a \rightarrow b$. E^4 , E^3 , etc., represent tetramers containing 4, 3, etc., phosphate groups, respectively; ● represents phosphorylated monomer units; ○, dephosphorylated units. Both a symmetrical and a nonsymmetrical 2-phospho-intermediate (E^2) are represented. Theoretical activity was calculated with the assumption that all partially phosphorylated intermediates are active in the presence of high G1P and inactive with G6P.

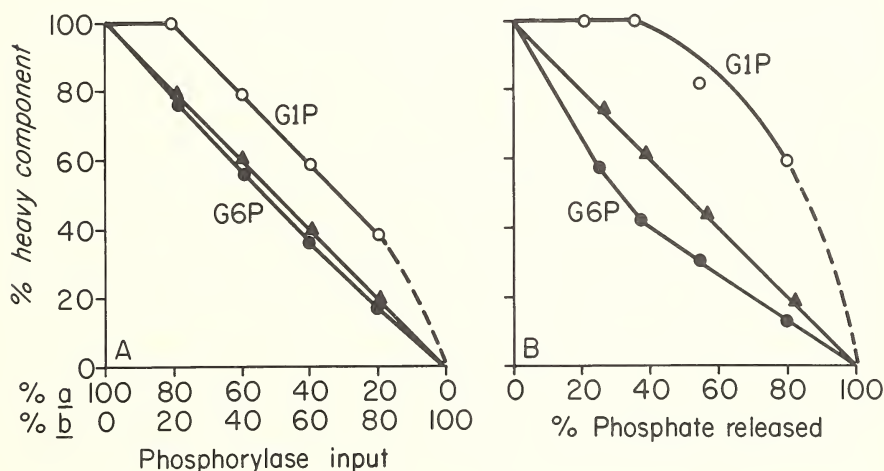


TEXT-FIGURE 3.—Computer analysis of conversion of phosphorylase $a \rightarrow b$, showing appearance and disappearance of each intermediate species.

Furthermore, their state of aggregation, as obtained by sedimentation velocity measurement in the ultracentrifuge, would parallel their enzymatic activity. Table 1 lists activity and sedimentation values obtained for phosphorylase *a* (measured at 0.1 M G1P, with and without 0.005 M G6P), during a phosphatase reaction, as compared to theoretical values calculated according to the proposed model. Good agreement can be found between the three sets of values.

PROOF OF EXISTENCE OF INTERMEDIATES

If partially phosphorylated intermediates were not produced in the phosphorylase *b*→*a* reaction, we would be dealing, at any one time, only with mixtures of phosphorylase *b* and *a*. To rule out this possibility, the properties of a solution obtained from a phosphorylase phosphatase reaction (presumably containing the postulated partially phosphorylated intermediates) were compared with artificial mixtures containing the same relative proportion of pure phosphorylase *b* and *a*. Sedimentation velocity measurements were made on both these mixtures; the results are illustrated in text-figure 4A and B, in which percent heavy component calculated by area analysis is plotted against the percent phosphate released (4B) or against relative proportions of phosphorylase *a* and *b* (4A). Total enzyme concentration in both instances was 6 mg/ml.



TEXT-FIGURE 4.—Ultracentrifuge behavior of samples obtained from a phosphatase reaction (B), as compared to mixtures of pure phosphorylase *a* and *b* (A). ▲ = control; ○ = with 0.1 M G1P; ● = 0.005 M G6P. All samples contained 0.2 M NaF to block the phosphatase reaction at various percentages of phosphate released.

TABLE 1.—Comparison of calculated and observed values for activity of the proposed model system

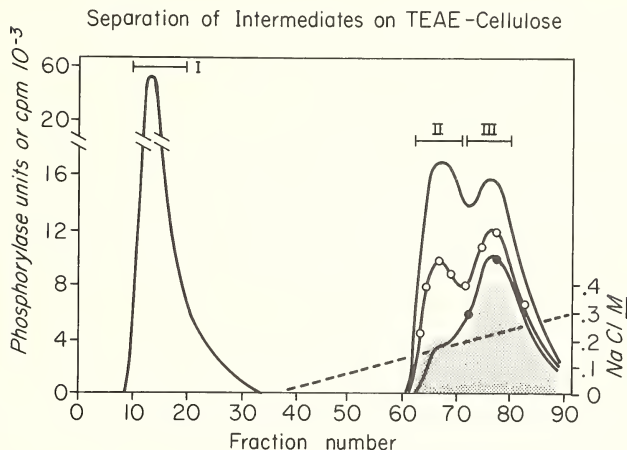
³² P released (%)	Activity expected for phosphorylase species (%)						Total theoretical activity or tetramer (%)	Observed	
								Activity (%)	Tetramer (%)
		E ⁴	E ³	E ²	E ¹	E ⁰			
25.....	Total	32	41	21	5	0			
	G1P	32	42	21	5	0	100	100	100
	G1P + G6P	32	21	5	0	0	57	56	56
50.....	Total	6	25	37	25	6			
	G1P	6	25	37	25	0	94	90	89
	G1P + G6P	6	1	9	0	0	27	31	32
75.....	Total	0	5	21	42	32			
	G1P	0	5	21	42	0	68	60	61
	G1P + G6P	0	2.5	5	0	0	7.5	10	14

It can be seen that, in both cases, addition of G1P caused a distinct increase in heavy component. This increase, however, is much more pronounced in the sample obtained from the phosphatase reaction than in the artificial mixture of pure phosphorylase *a* + *b*. Furthermore, the phosphatase sample shows an appreciable decrease in the percentage of heavy component in the presence of G6P, while none is observed in the mixture of phosphorylase *a* and *b*. The sedimentation patterns of pure phosphorylase *a* or *b* are unaffected by G6P. The effects of G1P, however, on the separate enzymes will be discussed. These experiments indicate that since the solution obtained from the enzymatic reaction behaves differently than corresponding mixtures of phosphorylase *a* and *b*, it must necessarily contain different molecular species.

Further confirmation of the existence of partially phosphorylated intermediates was provided by their separation on column chromatography. First attempts, using the products of a phosphatase reaction, were unsuccessful because the high concentration of NaF required to block the reaction interfered with the ion exchange chromatography. This difficulty was overcome by use of samples obtained from a phosphorylase *b*→*a* reaction mixture: This conversion requires Mg⁺⁺ and is blocked by addition of a slight excess of EDTA.

Text-figure 5 illustrates the elution pattern of a reaction mixture blocked at 15% conversion, following chromatography on TEAE-cellulose. Three fractions were obtained: a large pool of unreacted, unlabeled phosphorylase *b* (I), and two variously labeled fractions. Fraction II had a protein/³²P M ratio of 1.20 and a +G6P/−G6P activity ratio of 0.53, while values of 2.05 and 0.75, respectively, were obtained for Fraction III.

When Fraction II was isolated and resubjected to chromatography after standing for a few hours, two fractions were obtained: Again,



TEXT-FIGURE 5.—TEAE-cellulose chromatography of a $b \rightarrow a$ conversion reaction mixture blocked after 15% ^{32}P -incorporation by addition of 10^{-2} M EDTA. The sample was passed through a charcoal-Sephadex G-25 column prior to the ion exchange chromatography to remove salts and unreacted ^{32}ATP . Phosphorylase was measured in the direction of glycogen synthesis in the presence of 1% glycogen and 0.1 M G1P with the following additions: \circ = none (control); — = 10^{-3} M AMP; and \bullet = 10^{-3} M G6P.

totally unlabeled phosphorylase b appeared and a new labeled fraction with protein/ ^{32}P M ratio of 4 and +G6P/−G6P activity ratio of 0.94. Evidently, the partially phosphorylated intermediate present in Fraction II had undergone almost complete hybridization to phosphorylase b and a .

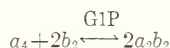
INTERACTIONS OF PHOSPHORYLASE a AND b

A partially phosphorylated complex can be produced by combination of phosphorylated subunits from phosphorylase a and nonphosphorylated subunits from phosphorylase b in the presence of G1P. Several characteristics of this reaction are worthy of note: 0.1 M G1P has only a small effect on the sedimentation properties of phosphorylase a or b . The effect of G1P on a mixture of phosphorylase a and b is to increase the amount of heavy component in the ultracentrifuge by approximately 20% at 20 C. In the absence of G1P, no change in the sedimentation behavior is observed; that is, the mixture has the same proportion of heavy and light components as the amounts of phosphorylase a and b put into the centrifuge cell. The effect of G1P on the mixtures is not time-dependent. Area distributions of the sedimenting peaks are the same immediately after mixing as after standing for 15 hours.

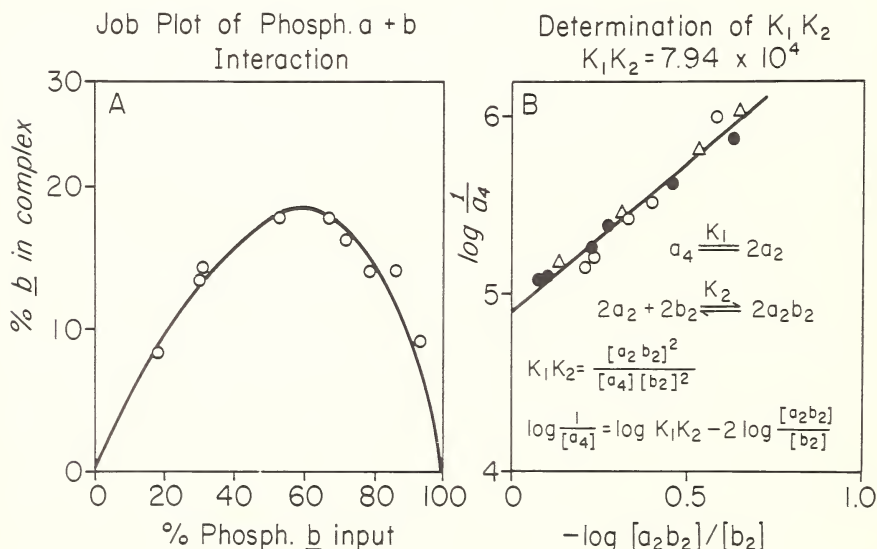
Upon dialysis of the G1P from the mixture, the system returns to the original proportion of heavy and light material, that is, the reaction is reversible. Another way of reversing or preventing the formation of the complex is by addition of G6P.

The method of continuous variations (14, 15) has been used for a study of the stoichiometry of the reaction. While this operation is normally carried out at constant total molarity of the reacting species, we have chosen to keep the total weight concentration constant. The equations for such a system are a bit more complex than for constant molar concentration but uncertainties concerning molecular weight are largely eliminated.

By measuring the amount of decrease in the slower sedimenting component as a function of composition, the stoichiometry of the complex has been determined to be a_2b_2 . Text-figure 6A shows a typical Job plot at 6.0 mg/ml total concentration. In text-figure 6B, three such plots have been combined to determine the equilibrium constant for the reaction



The solid curve in text-figure 6A is the theoretical curve drawn from the equilibrium constant determined in text-figure 6B. For such a



TEXT-FIGURE 6.—Job plot of complex formation in mixtures of phosphorylase $a + b$, in 0.1 M G1P, 20 C (A). Percent b in complex represents % total heavy component minus % phosphorylase a input. Open circles are observed values, in good agreement with the theoretical curve calculated from (B) for an a_2b_2 complex. Points from 3 different Job plots were used in determining the association constant K_1K_2 for a_2b_2 .

method of continuous variations on a weight basis, showing a maximum decrease of 20% in the phosphorylase *b*, the maximum percent complex formation should occur at 28% phosphorylase *b* put into the reduction mixture if the complex were a_3b , and 83% for ab_3 . The observed maximum at 60% phosphorylase *b* is exactly the theoretical value for only one complex of the a_2b_2 type.

Thus, independent of the phosphatase and kinase reactions, one can form a partially phosphorylated, enzymatically active hybrid of phosphorylase *a* and *b* whose properties are sensitive to G6P.

ROLE OF G6P IN THE CONTROL OF PHOSPHORYLASE ACTIVITY

A direct effect of G6P on the activity of phosphorylase had not been previously described. Morgan and Parmeggiani (16) reported that G6P, by competing with AMP, could inhibit the activation of phosphorylase by this nucleotide. In the work presented here, it is clear that G6P has a direct effect on the enzyme by dissociating the partially phosphorylated intermediates and rendering them inactive, even in the absence of any added nucleotide.

The site of action of G6P on phosphorylase is not known. On the one hand, one could assume with Morgan and Parmeggiani that G6P binds at the AMP binding site. A direct competition between G6P and AMP would explain a number of phenomena in which these two effectors act in diametrically opposite ways: release by G6P of the AMP-dependent activation of phosphorylase *b*, total inhibition of phosphorylase phosphatase, and tetramerization of *a* + *b* mixtures. In other instances, however, G6P and AMP have similar effects: Both these effectors prevent removal of pyridoxal 5'-phosphate from the enzyme (7) and block hybridization of Type I and III phosphorylases (17). Since these two latter phenomena appear to require monomerization of the enzyme, it would seem that dissociation of phosphorylase dimer is prevented by the sugar ester.

Of course, the work described here has only been carried out on isolated enzymes. Furthermore, because of technical difficulties, particularly in the ultracentrifuge studies, glycogen, a most important component of the phosphorylase reactivation, had to be omitted. Indications have already been obtained, however, that in crude muscle extracts or in a "glycogen fraction" isolated from rabbit muscle (and which appears to contain almost all the enzymes involved in glycogen synthesis and breakdown) the rapid and reversible activation of phosphorylase *b*, which is observed upon addition of Ca^{++} , Mg^{++} , and ATP, yields enzymatic species that are inhibited by G6P.

It is logical to assume, therefore, that the intracellular activation of phosphorylase does not always proceed to completion; that when

muscle contracts, for instance, phosphorylase *b* is not totally converted to phosphorylase *a*. If this assumption is correct, then partially phosphorylated intermediates will be produced whose enzymatic activity and structural properties are neither those of phosphorylase *b* nor *a*: These intermediates will show high activity in the presence of G1P, and will be almost completely inhibited by G6P. Clearly, variations in the relative proportion of these sugar esters may afford a sensitive and effective control of glycogen utilization.

RESUMEN

La actividad de la fosforilasa muscular es determinada y, en ocasiones, regida por un número diverso de factores que incluyen la fosforilación de la proteína (es decir, la conversión de la fosforilasa *b* en *a* por medio de la fosforilasa cinasa), la activación por el AMP, la presencia de piridoxal 5'-fosfato (PLP), el estado de agregamiento de la molécula, etc. En todos los casos participan regiones específicas de la molécula proteínica y es posible concebir al fenómeno de la regulación de la actividad enzimática como resultante de ciertas interacciones de sitios determinados.

Se aportan pruebas de que la actividad de la enzima puede ser controlada por otro parámetro adicional: cuando las fosforilasas *a* y *b* se interconvierten una en la otra, la fosforilación de la proteína no se lleva a cabo en una reacción de todo o nada como se había supuesto originalmente, sino que procede de una manera gradual en la que se producen intermediarios parcialmente fosforilados. Estos híbridos "fosfo-defosfo" muestran actividad catalítica y conductas fisicoquímica diferente a las fosforilasas *a* o *b*: poseen elevada actividad enzimática cuando se miden (en el sentido de la síntesis de glucógeno) a concentraciones elevadas de G-1-P, pero esta actividad se deprime de modo considerable por medio de cantidades pequeñas de G-6-P. Asimismo, su conducta de sedimentación, tal como se observa en la ultracentrífuga, se ve intensamente afectada por la presencia de ésteres de azúcares: la G-1-P aumenta la proporción del componente pesado (posiblemente el que corresponde al tetrámero), mientras que la G-6-P favorece la formación de la forma dimérica. Se presenta un modelo que puede explicar estos fenómenos en términos de la estructura molecular de la enzima y que es compatible con los resultados experimentales. También se ha estudiado la interacción de fosforilasas *a* y *b* puras en diversas condiciones y se aportan pruebas de la producción de un híbrido a_2b_2 . Se analiza el papel de estos intermediarios parcialmente fosforilados en la regulación de la actividad de la fosforilasa.

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Inhibition of Gluconeogenic Effects of Cortisol by Nucleotides and Related Substances^{1,2,3}

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SUMMARY

Free nucleotides in some enzymatic systems may affect the reaction rates in a way distinct from product inhibition or feedback effects. The effect of such nucleotides on some enzymes may be more general, and most of the nucleotides or their derivatives may affect several enzymatic systems. It is especially important that the turnover of nucleotides in the cell is very high, and their concentration could act as a regulator of enzymatic and metabolic processes. *In vivo* studies of some aspects of carbohydrate metabolism in adrenalectomized rats as modified by nucleotides and related substances showed that: 1) The injection of ribonucleic acid, free nucleotides, and especially a mixture of adenylic plus guanylic acid provoked a diminution of liver glycogen and blood glucose; 2) the glu-

coneogenic action of cortisol is inhibited in animals treated with free nucleotides, as well as a blocking effect in the incorporation of glucose and amino acids into glycogen; 3) tyrosine- α -keto glutarate transaminase, glucose-6-phosphatase, fructose-1,6-diphosphatase, pyruvate carboxylase, and phosphorylase in the liver of experimental animals showed no changes in their activity; 4) glycogen synthetase increased its activity several fold after injection of ribonucleic acid or free nucleotides. The suggestion is made that nucleotides produce a preferential utilization of glucose for glycogen formation, whereas gluconeogenic substrates are not used for glycogen formation but for total oxidation in the tricarboxylic acid cycle.—*Nat Cancer Inst Monogr* 27: 61–69, 1967.

IT IS KNOWN that several nucleotides, especially those derived from adenine, participate actively in the regulation of the enzymatic activity of various systems. In general, these effects may be divided in three main groups:

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² This paper was presented at the Tuesday morning session but was relocated due to changes in emphasis in the subject matter.

³ See Discussion of this paper conducted by Dr. J. P. Changeux, p. 165.

1) Those in which they act as feedback agents, such as the inhibition of aspartate transcarbamylase by means of cytidine triphosphate (CTP) as described by Gerhart and Pardee (1); inhibition of fructose-1,6-diphosphatase by AMP, reported by Taketa and Pogell (2); and the feedback control, both positive and negative, exerted by purine and pyrimidine nucleotides on carbamyl phosphate synthetase, which maintains a balance between the rates of purine and pyrimidine production, as was recently shown by Anderson and Meister (3).

2) A second group of effects, according to Atkinson's ideas (4, 5), are those in which nucleotides, mainly those related to adenine, participate in the production and liberation of energy as ATP. In this category can be included the role of AMP as a positive modifier of phosphofructokinase (6) and, at the same time, as an inhibitor of fructose-1,6-diphosphatase. These facts have led Krebs (7, 8) to propose a special role for AMP in the regulation of glycolysis and gluconeogenesis.

3) Another type of effect mediated through nucleotides may be unspecific, *i.e.*, not related to feedback effects or regulation of energetic ATP. It may be mediated through conformational changes more or less related to the integrity of active sites; such would be the case of glutamic dehydrogenase, which is inhibited by GTP and stimulated by AMP (9), and the stimulation with GTP and inhibition with AMP of monocarboxylic-L-amino acid dehydrogenase of alanine, valine, leucine, isoleucine, etc., as demonstrated by Tomkins (9). The usual interpretation of these facts is to consider such nucleotides as modifiers of the equilibrium between associated and dissociated forms of the enzyme, each of them responsible for a part of the enzymatic behavior. Another example of such unspecific activity of nucleotides is the report by Grisolia (10) that the heat stability of glutamine synthetase is decreased in the presence of ATP, but when Mg^{++} , glutamate, and other substrates are added, ATP is protective.

Both nucleotides and nucleic acids produce some remarkable metabolic responses; Dole (11) reported that RNA, AMP, and adenosine had antilipolytic action in adipose tissue *in vitro* similar to that produced by insulin, which caused a higher utilization of exogenous glucose through a striking increase in diglyceride synthesis.

It is apparent, then, that nucleic acids, or their derivatives, *i.e.*, nucleotides, nucleosides, and free bases, may act as metabolic regulators, through mechanisms possibly related to structural changes in enzymic proteins.

In this paper we present results obtained *in vivo* through the injection of RNA and nucleotides into adrenalectomized animals in relation to its effect on carbohydrate metabolism.

RESULTS AND DISCUSSION

Early experiments in our laboratory showed that the injection of RNA and nucleotide mixtures caused some changes in the distribution of carbohydrates in rats. Their effects in adrenalectomized rats were observed to decrease the amount of liver glycogen and prevent the increase of glycogen found in the liver of rats treated with cortisol. Of interest was the finding of such substances as RNA or nucleotides that could block some of the well-known effects of cortisol. We considered this group of compounds potentially important as metabolic regulators naturally present in the cell environment, as well as a useful tool for the study of the mechanism of gluconeogenic action(s) of cortisol. To explore the latter effect, some aspects of carbohydrate metabolism, such as the levels of liver glycogen, blood glucose, and the incorporation of labeled glucose and alanine carbon into liver glycogen were studied, as well as the enzymatic activity of some systems related to such phenomena.

Table 1 shows the results of the administration of RNA or a mixture of ribonucleotides in adrenalectomized rats. RNA produced a statistically significant decrease in liver glycogen concentration; the absolute decrease was relatively small due to the low glycogen level usually encountered in fasted, adrenalectomized animals. A clear response to cortisol was observed, but this was almost completely prevented when an injection of RNA was given simultaneously with cortisol administration. Only a moderate lowering of glycogen level was observed when nucleotide mixtures were used; but, nevertheless, a clear and significant inhibition of the cortisol effect appeared. The inhibitory effect against cortisol may be related to the administration of a mixture of AMP plus GMP, inasmuch as when the latter were replaced by UMP plus CMP no effect whatsoever could be observed.

The concentration of blood glucose was moderately low in adrenalectomized animals; injection of cortisol into adrenalectomized animals provoked an increase in blood glucose that reached normal values. Administration of either RNA or a nucleotide mixture, or a mixture of AMP plus GMP caused a marked diminution of blood glucose, but had no influence on the increase of blood glucose due to cortisol administration. However, a significant increase of this parameter was observed after the administration of cortisol and a mixture of UMP plus CMP.

A striking difference in the total radioactivity incorporated into liver glycogen after the administration of glucose-U-¹⁴C glucose was observed in the different groups. The highest figures were those of the cortisol-treated rats, and the lowest were those of the control animals.

TABLE 1.—Effect of RNA and derivatives on the carbohydrate metabolism of adrenalectomized rats with or without added cortisol*

Substance injected	Liver glycogen† (mg/g protein) mean ± SE	Blood glucose§ (mg/100 ml) mean ± SE	Incorporation of glucose-U- ¹⁴ C into liver glyco- gen (cpm/total liver) mean ± SE	Incorporation of DL-alanine-1- ¹⁴ C into liver glyco- gen (cpm/total liver) mean ± SE
Saline.....	6.3 ± 1.3 (23)	53.5 ± 4.7 (13)	75 ± 10.3 (6)	48 ± 10.6 (7)
RNA.....	1.6 ± 0.3 (5)	36 ± 4.1 (11)	298 ± 87.6 (6)	55 ± 7.2 (6)
Mixture of ribonucleotides..	3.3 ± 1.0 (8)	—	—	—
Cortisol.....	94.7 ± 10.1 (19)	75.0 ± 6.1 (15)	1367 ± 204.2 (7)	2050 ± 469 (7)
Cortisol + RNA...	7.5 ± 2.7 (5)	75.0 ± 3.5 (15)	240 ± 96 (5)	103 ± 34.7 (7)
Cortisol + mixture of ribonucleotides	5.8 ± 1.4 (10)	74.5 ± 3.4 (4)	—	—
Cortisol + AMP + GMP...	9.3 ± 4.5 (8)	61.6 ± 6.9 (4)	—	—
Cortisol + UMP + CMP...	64.8 ± 31.2 (7)	99.2 ± 8.1 (4)	—	—

*Experimental design: 120 g rats, adrenalectomized for 60 hours; deprived of food 5 hours before they were killed. Ribonucleic acid (RNA) from yeast and nucleotides (NBCoγ) suspended in saline solution at pH 7.3 for intraperitoneal injection, at a dose of 20 mg/100 g rat. Mixture of ribonucleotides is an equimolecular mixture of guanylic acid (GMP), adenylic acid (AMP), citidylic acid (CMP), and uridylic acid (UMP). Cortisol injected separately at the dose of 2.5 mg/100 g rat. Injections 3.5 hours before killing. Radioactive glucose (1 μc/100 g rat) 30 minutes, and radioactive alanine (1 μc/100 g rat) 2 hours before killing.

† Reference (12).

‡ Reference (13).

§ Reference (14).

|| Numbers in parentheses represent number of experimental animals.

The administration of RNA produces a fourfold increase in the total radioactivity incorporated in the liver, when compared to that in control rats. RNA injected into cortisol-treated rats prevents the increase in total radioactive incorporation and amounts almost to that in animals injected with RNA alone.

In relation to the incorporation of DL-alanine-1-¹⁴C into glycogen, results were similar to those obtained when labeled glucose was given; the alanine uptake into liver glycogen was markedly enhanced by cortisol and was prevented when RNA was administered in addition to cortisol. RNA alone did not modify significantly the incorporation found in control rats.

The inhibition by RNA of cortisol-dependent glycogen deposition may be caused by a blocking of the glycogen biosynthetic pathway or by an increase in glycogen degradation. This latter mechanism seems unlikely since a significant lowering of blood glucose level was consistently observed in RNA-injected control animals, and the blood glucose level of the RNA plus cortisol group resembled that observed in the cortisol group.

On the other hand, RNA or the active nucleotides show an antagonistic effect toward the glucogenic action of cortisol. Since the

increased rate of incorporation of labeled glucose and alanine into liver glycogen observed after cortisol administration is significantly diminished by RNA, and since RNA or nucleotides are not glycogenolytic agents, then its antagonistic effect on early cortisol-induced gluconeogenesis might be explained in terms of an interference with the primary metabolic modifications that lead to glycogen synthesis. Accordingly, experiments were performed to study the enzymatic activity of some critical steps concerned with the formation and degradation of glycogen and glucose, *e.g.*, phosphorylase, glycogen synthetase, glucose-6-phosphatase, fructose-1,6-diphosphatase, and pyruvate carboxylase activities; furthermore, since transaminases have been correlated with some aspects of carbohydrate metabolism (15), an appraisal of tyrosine α -ketoglutarate transaminase was made.

Table 2 shows the results of enzyme activity determinations in livers of adrenalectomized control rats and animals treated with RNA, cortisol, and a mixture of both RNA and cortisol. No significant changes were observed in the apparent activity of fructose-1,6-diphosphatase, and glucose-6-phosphatase under our experimental conditions, although Weber (22) has described a significant increase in such enzymatic activities after the repeated administration of cortisol. This

TABLE 2.—Enzyme activities in the liver of adrenalectomized rats treated with RNA and/or cortisol*

Enzyme	Saline	Substance injected		
		RNA	Cortisol	RNA + cortisol
Glucose-6-phosphatase†	2.81 \pm 0.26 (4)†	2.68 \pm 0.06 (5)	3.02 \pm 0.15 (5)	2.90 \pm 0.17 (4)
Fructose-1,6-diphosphatase§	0.59 \pm 0.01 (4)	0.58 \pm 0.02 (4)	0.60 \pm 0.01 (4)	0.61 \pm 0.01 (4)
Pyruvate carboxylase	2.2 \pm 0.65 (4)	1.86 \pm 0.17 (4)	1.60 \pm 0.11 (3)	1.67 \pm 0.13 (4)
Tyrosine- α -ketoglutarate transaminase¶	0.32 \pm 0.01 (9)	0.31 \pm 0.01 (8)	0.75 \pm 0.01 (4)	0.75 \pm 0.01 (4)
Phosphorylase**	0.13 \pm 0.02 (4)	0.18 \pm 0.01 (4)	0.18 \pm 0.05 (3)	0.15 \pm 0.01 (4)
Glycogen synthetase††	22.4 \pm 7.0 (4)	185.5 \pm 6.3 (4)	159.5 \pm 12.5 (4)	219.3 \pm 51.1 (4)

* Experimental conditions as in table 1; mean \pm SE.

† Numbers in parentheses represent number of experimental animals.

‡ Units: μ moles P/mg protein/hour (16).

§ Units: μ moles P/mg protein/hour (17).

|| Units: O.D./mg protein/minute (18).

¶ Units: μ moles/mg protein/hour (19).

** Units: μ moles P/mg protein/hour (20).

†† Units: μ moles UDP/g wet tissue/hour (21).

METABOLIC REGULATION

would imply that the effects obtained in our experiments are not related to possible modifications of these enzymes due to cortisol induction mechanisms.

Pyruvate carboxylase, an enzyme fully investigated by Utter (23) and considered by Krebs (8) as a critical step in the regulation of the reversal of glycolysis, showed no changes after cortisol and/or RNA administration.

In spite of the fact that fructose-1,6-diphosphatase and pyruvate carboxylase show no changes in their activity, as measured *in vitro*, they still can be considered as two sites for a feedback control of gluconeogenesis, as suggested by Krebs (8), inasmuch as their actual activity *in vivo* is not known.

Our investigation of tyrosine- α -ketoglutarate activity (table 2) confirms Litwack's findings (24) that RNA induction of this enzymatic activity in adrenalectomized animals depends on cortisol administration; besides, RNA, although having a clear blocking effect on the cortisol glucogenic action, does not prevent the transaminase induction. This situation is completely different from that observed by other authors, in which the gluconeogenic effect of cortisol was blocked by preventing enzyme induction with the usual protein synthesis inhibitors (25), and probably is more related to evidence (26, 27) indicating that the biosynthesis of gluconeogenic-inducible enzymes does not depend on the early metabolic response of glucocorticoid administration.

In the group receiving RNA, phosphorylase activity did not show any modifications, whereas glycogen synthetase increased sevenfold, this increase being similar to that observed in the cortisol group; the increase was not prevented when RNA and cortisol were administered simultaneously.

From the data on glycogen synthetase activity and total radioactive glucose incorporation into glycogen, we can assume that there is a stimulation of glycogen synthesis from glucose; but at the same time this contradicts the fact that glycogen concentration is low in animals treated with RNA alone or RNA plus cortisol.

Since cortisol gluconeogenic activity is inhibited by RNA, or by AMP plus GMP (*see* table 1), it is likely that, although glycogen formation from glucose is increased, the net effect is a lowering of total glycogen. This implies a blocking of some enzymatic steps in the gluconeogenic pathway, since an activation of glucogenolysis has been ruled out. That this can be the case is supported by some related findings: Krebs (28) has reported that AMP is an inhibitor of gluconeogenesis from lactate, and 5'-GMP is, according to Chang (29), an inhibitor of phosphoenolpyruvate formation from oxaloacetate.

There is no problem concerning the utilization of readily oxidizable carbohydrate substrates; some preliminary experiments from our laboratory show conclusively that RNA increases the endogenous oxygen uptake, pointing to a normal or elevated substrate consumption at the citric acid cycle level. This resembles Dole's (11) findings in adipose tissue, where RNA causes an increase in glucose utilization by decarboxylation in the hexose monophosphate shunt or by an elevation in glycerol and diglyceride synthesis. Hence, it is possible that nucleotides, mainly AMP and GMP, cause a preferential utilization of the intact glucose molecule for glycogen formation as well as for fatty acid synthesis, whereas potentially gluconeogenic substrates are predominantly oxidized in the tricarboxylic acid cycle and are not used as carbohydrate-forming materials.

With the experimental data at hand it is not possible to make a definite statement on the mechanism of action of this group of substances. It is important that the turnover of nucleotides in the cell is very high and that a modification of the nucleotide "pool" could change the metabolic rates or produce a deviation in a normal metabolic pathway through the activation or inhibition of some enzymes involved in the process.

RESUMEN

Se ha demostrado, en ciertos sistemas enzimáticos, que los nucleótidos libres pueden afectar la velocidad de la reacción por un camino diferente al de la inhibición por productos o por retroalimentación. Es posible que el efecto de los nucleótidos sobre algunas enzimas sea de tipo más general y que buena parte de los nucleótidos o sus derivados puedan afectar varios sistemas enzimáticos. Es de importancia reconocer que el recambio de los nucleótidos en el interior de las células es muy elevado y que su concentración podría participar en la regulación de los procesos enzimáticos y metabólicos.

Los estudios realizados *in vivo* sobre ciertos aspectos del metabolismo de los carbohidratos en ratas adrenalectomizadas, tal como se modifican por los nucleótidos o sustancias conexas, demostraron que: 1) la inyección de ácido ribonucleico, nucleótidos libres, y especialmente una mezcla de ácido adenílico más ácido guanílico produce una disminución del glucógeno hepático y de la glucosa sanguínea; 2) se observa una inhibición de la acción gluconeogénica del cortisol en animales tratados con nucleótidos libres, así como un efecto de bloqueo en la incorporación de glucosa y aminoácidos al glucógeno; 3) la transaminasa tirosina α -ceto glutárica, la glucosa-6-fosfatasa, la fructosa-1, 6-difosfatasa, la piruvato carboxilasa, y la fosforilasa en el hígado de los animales experimentales no mostraron ningún cambio en su actividad; 4) la glucógeno sintetasa aumentó su actividad varias veces después de la inyección de ácido ribonucleico o de nucleótidos libres.

Se sugiere que los nucleótidos producen una utilización preferente de la glucosa hacia la formación de glucógeno mientras que los sustratos gluconeogénicos no se utilizan para la formación de glucógeno sino que son llevados a su oxidación total es el ciclo de los ácidos tricarboxílicos.

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Inhibition of Skeletal Muscle Phosphorylases *a* and *b* by 1,2,4- α -Dinitrophenol^{1,2}

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SUMMARY

The enzymatic system phosphorylase *a*-phosphorylase *b* has an important role in the regulation of glycolysis in muscle. Kinetics of inhibition by 1,2,4- α -dinitrophenol (DNP) of phosphorylase *a* and *b* were studied with the crystalline forms of both enzymes obtained from rabbit skeletal muscle. DNP was found to be an inhibitor of phosphorylase *b* in a competitive way with 5'-AMP. Phosphorylase *a* was not inhibited at all, when recently crystallized. However, if phosphorylase *a* was crystallized from Versene and aged, inhibition by DNP started to appear, being protected by 5'-AMP. On the other hand, no inhibition was apparent if the enzyme was crystallized from cysteine. These results can be extended to muscle extracts; when they are assayed immediately, no inhibition of phosphorylase *a* by DNP was obtained.

As judged by experiments of dialysis equilibrium against bromothymol blue in the presence of 5'-AMP, DNP avoided the association of the protein with dye. DNP competed with the allosteric interaction of 5'-AMP with the protein, which causes the optimum configuration necessary to the enzymatic activity of phosphorylase *b*. The physiological implications of these observations seem very interesting in rats poisoned with DNP. Since phosphorylase *b* is inhibited by DNP, it is reasonable to assume that phosphorylase *a* is the active enzyme that participates in the muscles when they have the ability to contract. The transformation of phosphorylase *b* to *a* is not essential for increased glycogenolytic flux.—*Nat Cancer Inst Monogr* 27: 71-80, 1967.

HAAS (1), determining the glycogen content of muscle of rats under the influence of drugs, observed that after injection of 1,2,4- α -dinitrophenol (DNP) the concentration of glycogen decreased from 0.51 to

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. H. A. Lardy, p. 125.

0.25%. Also, the uptake of inorganic phosphorus (Pi) occurring in the process of phosphorylation of the glycogen in the muscle extract was considerably reduced in 1 hour by DNP (18.2 mg%) as compared with that of normal rats (65.1 mg%).

Ther and Müller (2), investigating the role of the adrenal glands in glycogen phosphorolysis, measured the phosphorus consumption in muscle extracts of normal and adrenalectomized rats and found no difference. On the other hand, rats poisoned with DNP had a definite decrease of Pi consumption in muscle extracts during glycogen phosphorylation. The isolated frog gastrocnemius poisoned with DNP had a fatigue curve similar to the normal. They therefore concluded that no obligatory correlation seems to occur between adynamia and disturbance of glycogen phosphorolysis.

Cori and Illingworth (3) have investigated the effect of glycogenolytic agents, such as DNP, on phosphorylase *a* content of frog sartorius muscle. Addition of DNP did not lead to a rise in phosphorylase *a*. When the phosphorylase *a* content in the muscle was high, there was a definite drop in phosphorylase *a* and an increase in lactate production. Glycogenolysis can apparently be accelerated at very low levels of phosphorylase *a*, without an increase in the active enzyme.

In view of this background we believed it was desirable to study the inhibition by DNP of the pure forms of phosphorylases *a* and *b* to have a better understanding of the facts given above. Our data on the kinetic behavior of DNP relevant to the activity of these enzymes are presented.

MATERIALS AND METHODS

Phosphorylases *a* and *b* were recrystallized at least 4 times according to Cori *et al.* (4) and Fischer *et al.* (5). The enzymes were freed of AMP either by treatment with Dowex-1 and Norit A (6) or by dialysis in Sephadex G-25 equilibrated with 10^{-3} M 2-mercaptoethanol, 1.5×10^{-3} M EDTA, and 10^{-2} M sodium β -glycerophosphate, pH 6.7.

Rabbit glycogen was prepared according to Manners and Ryley (7) and treated with Dowex-1 and Norit A to remove the AMP.

The crystalline enzymes were assayed according to the method of Cori *et al.* (4), using the enzyme diluted conveniently in either 0.04 M cysteine or 0.0015 M EDTA in β -glycerophosphate, pH 6.7.

The basic assay system contained (final concentration) in a total volume of 1 ml: 0.005 M β -glycerophosphate buffer, pH 6.7, with 0.015 M cysteine or 0.0025 M 2-mercaptoethanol, 0.005 M EDTA, 0.5% egg albumin, 0.5% glycogen, phosphorylase either *a* or *b* in suitable quantities, 0.016 M glucose-1-phosphate, and 0.001 M AMP for phosphorylase *b*. The reaction was started by addition of substrate. After incuba-

tion for 0, 10, and 20 minutes at 37 C, it was stopped by adding aliquots of 0.2 ml to 10% TCA. After centrifugation, a sample of the supernatant was withdrawn for the determination of Pi. The Pi produced was determined by the method of Fiske and Subbarow modified by Lohmann and Jendrassik (8). The units of phosphorylase were defined according to the method of Cori *et al.* (4).

Estimation of glycogenolytic activity of phosphorylase in muscle extracts.—In most experiments the glycogenolytic activity was measured by the determination of the amount of Pi consumed after addition of glycogen to the muscle extracts. Adult rats weighing about 200 g were killed by decapitation, and muscle from the legs and back was immediately removed and ground with sand in an ice-cold solution containing sodium fluoride (3.8%) and EDTA (0.08 M), pH 6.7, unless otherwise stated in the results. After centrifugation, 0.5 ml of the extracts was incubated for 30 minutes at 37 C with DNP and AMP in different concentrations. The reaction was started by addition of glycogen (final concentration 0.5%). The total volume was 1 ml. Aliquots of 0.25 ml were removed at 0, 10, and 20 minutes and added to 4 ml of 10% TCA. After centrifugation, the Pi was determined in the supernatant.

Blood lactic acid determination.—We determined lactic acid according to Sigma Technical Bulletin No. 825-UV using lactic dehydrogenase crystallized from beef heart (9). The animals were anesthetized with ether, and 1 ml of blood was taken from the femoral artery and withdrawn in 1 ml of 6% perchloric acid. After centrifugation, samples of 0.1 ml were diluted to a final volume of 3 ml with a solution containing 0.2 M glycine, 0.2 M semicarbazide, 0.0025 M NAD, and 100 μ g lactic dehydrogenase, pH 10.0. After 1 hour of incubation at 37 C, the optical density was determined at 340 m μ .

Dialysis equilibrium (10).—Dialysis equilibrium experiments were performed on a Sephadex G-25 (fine) column (60 \times 1 cm) pre-equilibrated with 10^{-3} M 2-mercaptoethanol, 1.2×10^{-3} M EDTA, 10^{-2} M β -glycerophosphate, 1.8×10^{-5} M bromothymol blue (BTB), AMP, and DNP as stated in "Results." Phosphorylase *b*, at least 4 times crystallized and free of AMP, was run through the Sephadex column at a rate of 0.3 ml/minute. Aliquots of about 2 ml of eluate were taken and were made alkaline with 0.1 ml of 3 M NaOH prior to measuring optical density at 620 m μ , by use of 2.25×10^{-4} as the molecular absorption coefficient (11). The protein concentration of the samples was determined at 280 m μ , and the dye bound to protein was calculated after the correction of the volume.

RESULTS

In initial experiments, optimum conditions were established for the time of DNP action *in vivo*. Two and a half mg DNP/100 g of rat injected intraperitoneally was enough to produce an effect in 30 minutes. Thus, groups of animals were used for glycogen phosphorylation and lactic acid determination. The rats of both groups (controls and those given injections of DNP) were killed after anesthesia with ether, and the muscle and blood were removed rapidly. Muscles were weighed and ground with sand in the solution of fluoride-EDTA at 0 C, pH 7.0 (1 volume/g muscle). The uptake of inorganic phosphorus in 15 minutes was 50 mg% (± 6) for the normal rats and 23 mg% (± 4) for the poisoned ones. The samples of blood were withdrawn into 6% perchloric acid for lactic acid determinations. Table 1 shows the results.

We assayed the activities of phosphorylase in the muscle extract in the direction of synthesis of glycogen, using glucose-1-phosphate in the presence of 10^{-3} M DNP. In one typical experiment the activity without AMP decreased in 15 minutes from 647 units/ml to 413 units, and with AMP from 3410 units/ml to 2000 units.

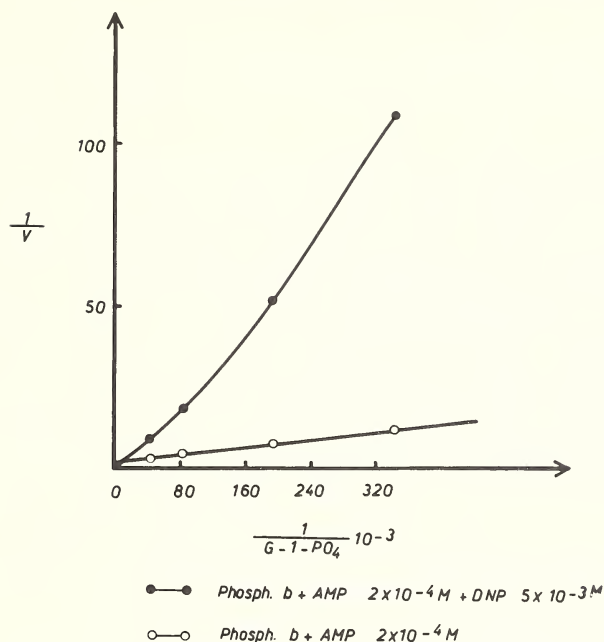
When crystalline phosphorylases *a* and *b* were assayed to test the inhibition by DNP, we were surprised to verify that only phosphorylase *b* was inhibited. No inhibition of phosphorylase *a* occurred even when a higher concentration of DNP such as 10^{-2} M was used.

TABLE 1.—Blood-lactic acid determination in normal rats and in rats given injections of DNP (2.5 mg/100 g)

Rats	Normal				DNP			
Lactic acid (μ mole/ml).....	3.18	1.93	1.74	5.01	11.69	4.34	8.68	8.78
	1.13	1.93	2.51	2.89	7.14	7.24	5.41	5.41
	2.65	4.54	3.86	4.54	5.41	9.17	6.46	5.60
	4.25	2.89	2.32	3.19	3.52	7.14	5.41	5.41
	1.54	2.70	4.06	2.46	3.43	6.08	6.28	5.89
Average.....	2.96 ± 1.10				6.42 ± 1.89			

To establish the mechanism of inhibition of phosphorylase *b* by the DNP, the initial rate was measured with glucose-1-phosphate as substrate. Phosphorylase *b* was incubated with different concentrations of substrate at a constant level of 2×10^{-4} M AMP in the presence or absence of 5×10^{-3} M DNP. A double reciprocal plot was used to present data, according to Lineweaver and Burk (text-fig. 1).

As can be seen, the inhibition is higher when the substrate concentration decreases. Increasing the concentration of glucose-1-phosphate will relieve the inhibition of DNP, but in this instance the kinetics are

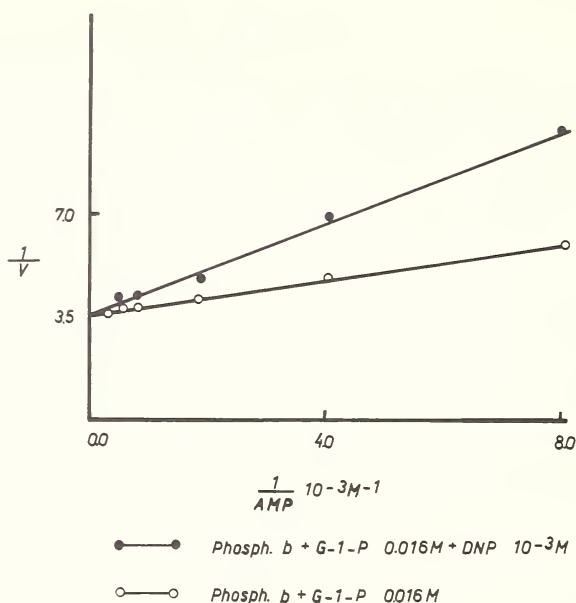


TEXT-FIGURE 1.—Inhibition of phosphorylase *b* by DNP: ○ — ○ = phosphorylase *b* + AMP $2 \times 10^{-4} M$; ● — ● = phosphorylase *b* + AMP $2 \times 10^{-4} M$ + DNP $5 \times 10^{-3} M$.

not the competitive type. Text-figure 2 also shows the double reciprocal plot resulting from performing the experiment the same way as described in text-figure 1. Different concentrations of AMP were used and the concentration of 0.016 M glucose-1-phosphate was kept constant. The inhibition by DNP follows strictly competitive kinetics with respect to the concentration of AMP.

The results obtained by Ullmann *et al.* (10) and Madsen (12), showing the allosteric effect of AMP on the phosphorylase *b*, as well as the analysis of text-figure 1, suggested that we should carry out experiments to investigate whether DNP would abolish the allosteric effect of AMP.

Table 2 shows experiments of dialysis equilibrium in Sephadex G-25 column, pre-equilibrated with $1.8 \times 10^{-5} M$ BTB. Purified phosphorylase *b* with BTB in the same concentration was allowed to be dialyzed through the column to equilibrium. After that, the enzyme with $10^{-3} M$ AMP was dialyzed in a column pre-equilibrated as usual, but with AMP in the same concentration. Finally, the enzyme in the presence of $10^{-3} M$ AMP plus $5 \times 10^{-3} M$ DNP was dialyzed in the same column but contained AMP and DNP. About 10 mg of enzyme was used each time and the bound phosphorylase *b*-BTB was determined as described.



TEXT-FIGURE 2.—Inhibition of phosphorylase *b* by DNP: ● —● = phosphorylase *b* + glucose-1-phosphate (G-1-P) 0.016 M + DNP 10^{-3} M; ○ —○ = phosphorylase *b* + glucose-1-phosphate 0.016 M.

TABLE 2.—Effect of DNP on the association of BTB with phosphorylase *b**

Additions	Molars of BTB/molar of phosphorylase <i>b</i> (M. W. 250,000)
None.....	1.15
AMP 10^{-3} M.....	3.05
AMP 10^{-3} M + DNP 2.5×10^{-3} M.....	1.25

*Ten mg of phosphorylase *b* was dialyzed to equilibrium through the Sephadex column (see text).

We have confirmed the results of Ullmann *et al.* (10), that AMP changes the configuration of phosphorylase *b* and induces the appearance of three loci to bind BTB as compared with 1 locus in the enzyme without the coenzyme. The same table shows that DNP prevents that change of configuration, since the number of loci for BTB remains the same as that of the enzyme in the absence of AMP.

DNP also competes with AMP in the crystallization of phosphorylase *b*. Thus, when phosphorylase *b* (4 times recrystallized) was recrystallized in the presence of 0.03 M cysteine, 0.01 M magnesium acetate, and 10^{-3} M AMP, pH 6.7, at 0 C procedure, the crystals started to appear after 30 minutes. When the same procedure was performed in the presence of 5×10^{-3} M DNP, there was a delay in the process of

crystallization of up to 180 minutes. When we used a higher concentration of DNP, such as 10^{-1} M, it was not possible to get crystals unless a concentration of AMP of the same magnitude as DNP was added simultaneously.

Experiments in crude extracts occasionally showed a certain inhibition of phosphorylase *a* by DNP that disappeared with the purification of the enzyme, as we have already stated. After several tentatives it was found that the inhibition was a function of the "aging" of the phosphorylase *a* in the extracts. Even the enzyme crystallized from EDTA maintained at 0 C suffered an inhibition by DNP with aging that was reversed by AMP. On the other hand, no inhibition was apparent if the enzyme was crystallized from cysteine. Table 3 shows the results. The experiment was performed by incubation of phosphorylase *a* with 10^{-2} M DNP for 20 minutes. Once this fact was known, all the muscle extracts were assayed immediately and no inhibition of phosphorylase *a* could be obtained.

TABLE 3.—Effect of DNP on aged phosphorylase *a* crystallized from EDTA and cysteine

Aging of phosphorylase <i>a</i> at 0 C	Crystallized in EDTA		Inhibition (%)	Crystallized in cysteine		Inhibition (%)
	Control (units of phospho- rylase)	+ DNP (units of phospho- rylase)		Control (units of phospho- rylase)	+ DNP (units of phospho- rylase)	
Fresh crystallized.....	8,440	8,440	0	17,200	17,200	0
2 days after.....	8,720	6,840	21.5	17,000	17,100	0
4 days after.....	10,240	6,360	38.0	—	—	—
6 days after.....	8,440	4,840	42.5	—	—	—
20 days after.....	8,880	4,960	44.2	11,600	11,600	0
20 days after + AMP 10^{-3} M.....	8,880	8,870	0	—	—	—
New crystallization...	11,000	11,000	0	18,000	18,000	—

To prove that only phosphorylase *b* is inhibited in muscle extract, we performed the following experiments. The muscle of the rats was rapidly homogenized with water in a proportion of 0.5 ml of cold water to 1 g of muscle and incubated either with water or 0.008 M ATP and 0.006 M calcium acetate, pH 7.0. After 30 minutes the extracts were diluted twice with sodium fluoride, 3.8%, and 0.08 M EDTA to stop the interconversion of phosphorylase *b* into *a*, and the rate of glycogen phosphorylation was determined. Table 4 shows the results. During the treatment with Ca^{++} and ATP, it was possible to transform some phosphorylase *b* to *a* (5). The ratio phosphorylase-AMP/phosphorylase + AMP $\times 100$ which was 10 became equal to 32 after treatment. Thus, in muscle which contains predominantly phosphoryl-

ase *b*, the inhibition by 10^{-3} M DNP is about 40%; 10^{-2} M AMP was able to overcome the inhibition. The inhibition of phosphorylase *a* was insignificant.

DISCUSSION AND SUMMARY

The analysis of the results shows that DNP inhibits crystalline phosphorylase *b* by competition with AMP. Phosphorylase *a* is not inhibited except when crystallized from Versene and aged. The competition mechanism, as judged by dialysis equilibrium against BTB, seems to be that DNP hinders the allosteric effect of AMP on phosphorylase *b*. DNP competes also with AMP in decreasing the velocity of the process of crystallization of phosphorylase *b*. In muscle extracts the "inhibition" of phosphorylase *a* seems to be due to either the presence of AMP in the extracts or the relatively long interval between the preparation of extract and the assay. When the assay is performed soon after the preparation of the homogenate, phosphorylase *a* is not inhibited, even when incubated with DNP 10^{-2} M for 30 minutes.

TABLE 4.—Inhibition of phosphorolysis of glycogen by DNP in muscle extracts

Extracts and additions	Pi consumed (mg)*
Extract with water.....	4.30
+ AMP 10^{-3} M.....	43.70
+ AMP 10^{-3} M + DNP 10^{-3} M.....	27.20
+ AMP 10^{-2} M.....	49.20
+ AMP 10^{-2} M + DNP 10^{-3} M.....	48.90
Extract with ATP and Ca^{++}	14.90
+ DNP 10^{-3} M.....	14.60
+ AMP 10^{-2} M.....	50.00
+ AMP 10^{-2} M + DNP 10^{-3} M.....	49.00

*The Pi is expressed in mg of Pi consumed per 100 ml of extract in 15 minutes at 30 C.

The findings described in the introduction may be explained on the basis that only phosphorylase *a* is important for glycogen phosphorolysis in the skeletal muscle in the animals given injections of DNP. Despite the inhibition of phosphorylase *b* by DNP, the muscles are still able to carry out activities at normal levels in the presence of the drug. This situation could be compared with that of the strain of mice which apparently lacks phosphorylase *b* kinase in muscle and where no significant rise in the level of phosphorylase *a* could be demonstrated after 10 seconds of tetanus, whereas in normal muscle the phosphorylase *a* content rose to 70% of the total (13). This example illustrates that the phosphorylase *b*→*a* transformation is not essential

for increased glycogenolytic flux (14). Furthermore, since DNP is an activator of adenosinetriphosphatase (15), the process of $b \rightarrow a$ transformation could be affected in the poisoned animal. Discussing the role of phosphorylase b during the stimulation of frog sartorius muscle, Helmreich and Cori (14) discarded a possible activation of the enzyme since the measurements of 5'-AMP concentration in muscle at different rates of stimulation gave no support to such a mechanism.

RESUMEN

El sistema enzimático fosforilasa a -fosforilasa b tiene un papel de importancia en a regulación de la glucólisis en el músculo.

La cinética de la inhibición de las fosforilasas a y b por el 1,2,4- α -dinitrofenol (DNF) se estudió con las formas cristalizadas de ambas enzimas preparadas a partir de músculo esquelético de conejo. Se encontró que el DNF es un inhibidor de la fosforilasa b en forma competitiva con el 5'-AMP. La fosforilasa a no es inhibida en lo absoluto, cuando se emplea una preparación recientemente cristalizada; sin embargo, si la fosforilasa a es cristalizada de verseno y envejecida, empieza a aparecer inhibición DNF que es protegida por medio de 5'-AMP. Por otro lado no se encuentra la menor inhibición si la enzima es cristalizada de cisteína. Estos resultados pueden ampliarse a los extractos musculares: si se hace el ensayo de inmediato no se encuentra inhibición de la fosforilasa a por medio del DNF.

Sobre la base de los experimentos de equilibrio por diálisis contra azul de bromotimol en presencia de 5'-AMP, el DNF impide la asociación de la proteína con el colorante. El DNF establece competencia con la interacción alostérica del 5'-AMP con la proteína, lo que causa un óptimo de la configuración necesaria para la actividad enzimática de la fosforilasas b .

Las implicaciones fisiológicas de estos hechos son muy interesantes en las ratas intoxicadas con DNF. Como la fosforilasa b es inhibida por el DNF es posible suponer que la fosforilasa a es la enzima activa que participa en los músculos cuando éstos tienen la propiedad de contraerse. La transformación de la fosforilasa b en la a no es esencial para obtener un aumento del camino glucogenolítico.

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Inhibition of GSSG Reductase by a Mixed Disulfide Complex of CoASSG^{1,2}

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SUMMARY

There is a NADPH-dependent CoASSG reducing activity in the commercially purified yeast GSSG reductase and in the partially purified GSSG reductase from yeast and rat liver. The GSSG reductase activity from these sources can be inhibited by the mixed disulfide complex (CoASSG) when the incubation is carried out in the presence of NADPH. The inhibitory effect can be partially reversed by dialysis. If the enzyme is incubated with its substrate (GSSG) and CoASSG, in the absence of NADPH, the inhibition effect cannot be demonstrated. Due to the break-

down of CoASSG, the inhibition is less accentuated with the partially purified extracts from yeast and rat liver. EDTA enhances the CoASSG and GSSG reductases in purified yeast preparation, but shows no effect on CoASSG reducing activity of rat liver. The mechanism of inhibition is discussed according to the observation by Massey and Williams that NADPH first reduces an active center disulfide of the GSSG reductase, which in our case permits the formation of a protein-SS-CoA complex.—*Nat Cancer Inst Monogr* 27: 81-88, 1967.

WE HAVE BEEN able to isolate from rat liver and to characterize a mixed disulfide complex formed by coenzyme-A and glutathione (1, 2). Independently, Chang and Wilken (3) have identified it in bovine liver. This compound seems to be a normal component of rat and bovine liver and yeast which can be formed as follows:



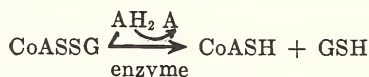
In order to gain insight into the role of this compound in the liver cell, a new enzymic activity which might metabolize this complex in

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² See Discussion of this paper conducted by Dr. H. A. Lardy, p. 125.

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the presence of a hydrogen coenzyme donor was investigated according to the following reaction:



We think that this reaction [2] must be necessary in order to open specifically the disulfide bridge, releasing CoASH and GSH to the free state within the cell. The enzymic activity has been shown to be present in rat liver and yeast extracts and works in the presence of NADPH₂ or NADH₂ (4). Reversal of the reaction in [2] did not occur after incubation of CoASH and GSH in the presence of enzyme and NADP⁺.

An additional finding which we would like to report here is that the CoASSG inhibits the commercially purified yeast GSSG reductase and the partially purified (40–60% ammonium sulphate saturation fraction) GSSG reductase from yeast and rat liver obtained in this laboratory. The inhibition can be accomplished only when the enzyme is incubated with CoASSG in the presence of NADPH₂, but not if this hydrogen donor is absent during the incubation period. The inhibition can be eliminated by dialysis, and the activity of GSSG reductase is partially restored.

MATERIALS AND METHODS

CoASSG synthesis and purification.—The CoASSG was prepared *in vitro* by reacting 40 μmole of CoASH with 160 μmole of GSSG for 3 hours in 1 ml of 0.05 M sodium phosphate buffer (pH 6.8) in the presence of O₂ at 36 C. The incubation mixture was resolved by column chromatography in a Dowex-1-chloride column, 1.2 × 20 cm, according to Cohn, as previously described (2).

The UV absorbing peak eluted from the column with 0.01 N HCl-0.03 M NaCl was concentrated on charcoal and eluted with ethanol-water. The ethanol-water concentrated fraction was applied on Whatmann 3 MM filter paper for separation by high-voltage electrophoresis (5 kv, with a buffer of formic acid-acetic acid-water 1:4:45 v/v, pH 2.0) for 1.30 hours. After elution of the UV-absorbing and ninhydrin-positive component it was separated further by low-voltage paper electrophoresis in a 0.1 M citrate buffer (pH 3.2) with a current of 0.5 ma/cm (375 v) for 5 hours at 4 to 6 C. The area corresponding to CoASSG was eluted with water and resolved again by high-voltage electrophoresis before use in order to remove residual citrate.

Identity of CoASSG.—The CoASSG obtained, as described above, gives, after performic acid treatment and acid hydrolysis, the same components as those produced from the rat liver complex and has

the same chromatographic and ionophoretic characteristics; it has a UV-absorbing spectrum with a maximum at 257 m μ (2).

Enzyme Preparations

a) The purified yeast GSSG reductase (E.C.1.6.4.2.) was obtained from Boehringer Mannheim, a crystalline suspension in 2.8 M ammonium sulphate solution (1 mg/ml). Specific activity: approximately 70 units/mg.

b) The partially purified yeast GSSG-reductase (40–60% ammonium sulphate saturation) was obtained in this laboratory during the logarithmic phase of growth of cultivated baker's yeast (*Saccharomyces cerevisiae* St. Geb. Mayer ATCC 1946), utilizing the culture media according to De Kloet *et al.* (5); the packed yeast cells obtained by centrifugation were suspended in 0.1 M phosphate buffer, pH 6.2, and disrupted by sonication with an MSE sonicator for 15 minutes at 4 C; the opalescent liquid was centrifuged at 18,000 $\times g$ for 10 minutes at 4 C; and the supernatant was fractionated with ammonium sulphate.

c) The partially purified rat liver GSSG-reductase (40–60% ammonium sulphate saturation) was prepared from normal rat liver extracts as previously described (4).

Protein determination.—It was obtained by the Lowry method as described by Layne (6) using bovine albumin A grade (Cal Biochem) as standard.

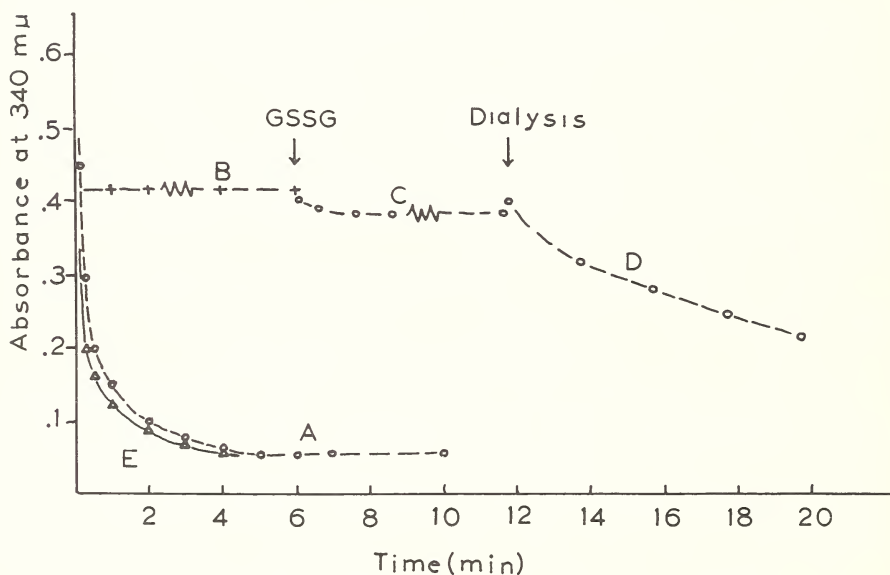
Glutathione and CoASSG reductase assay.—Glutathione and CoASSG-reductase activities were measured spectrophotometrically by recording the rate of decrease at 340 m μ . All determinations were made at room temperature with cuvettes having a 1 cm light path in a DK-2 Beckman automatic spectrophotometer or with a Zeiss spectrophotometer.

The incubation mixtures were made in a final volume of 1 ml of 0.068 M sodium phosphate buffer, pH 6.8 (unless otherwise specified), containing either 750 m μ mole of GSSG or 51 m μ mole of CoASSG, and approximately 75–100 m μ mole of NADPH. In some experiments a phosphate buffer containing 0.001 M EDTA was used. The enzyme activity is expressed in enzyme units per mg of protein according to Webb (7).

RESULTS AND DISCUSSION

As we have previously shown there is little activity of CoASSG reductase (0.137 U) in the commercial purified yeast GSSG reductase when compared to its normal activity under the conditions reported before (4). During the study of this activity it was noticed that the

yeast glutathione reductase could be largely inhibited if the enzyme was previously incubated with the CoASSG in the presence of NADPH for 10 minutes. In text-figure 1 the normal GSSG- splitting activity of yeast glutathione reductase is present (*curve A*). If the same amount of enzyme was incubated with CoASSG in the presence of NADPH in sodium phosphate buffer (*pH* 6.8), no appreciable breakdown of the mixed disulfide under these conditions could be observed (*curve B*). When the same preparation received, after a period of 10 minutes, 750 $m\mu$ mole of GSSG, the expected glutathione-reducing activity was completely inhibited (*curve C*). The inhibition can be partially eliminated with a 60 minute dialysis with phosphate buffer (*curve D*). Fur-



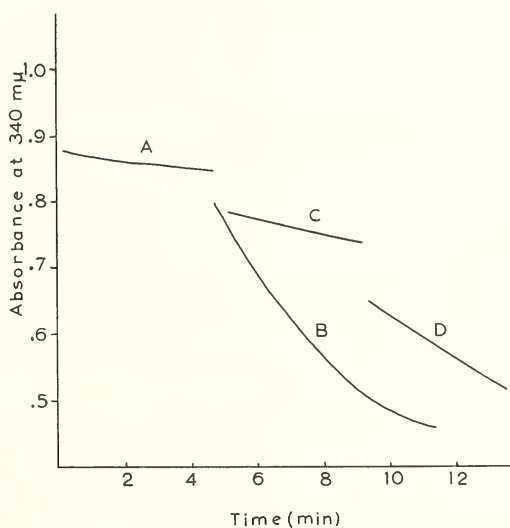
TEXT-FIGURE 1.—The CoASSG inhibition effect on purified yeast GSSG reductase.

(A) 900 μ liter of 0.068 M phosphate buffer (*pH* 6.8); 100 μ liter of GSSG (750 $m\mu$ mole); 10 μ liter of NADPH (\pm 75 $m\mu$ mole); and 10 μ liter of diluted 1:10 commercial purified yeast glutathione reductase (1 μ g of protein). (B) 900 μ liter of 0.068 M phosphate buffer (*pH* 6.8); 100 μ liter of CoASSG (51 $m\mu$ mole); 10 μ liter of NADPH (\pm 75 $m\mu$ mole); and 10 μ liter of diluted 1:10 commercial purified yeast glutathione reductase (1 μ g of protein). (C) The same incubation mixtures of B after 10 minutes of incubation, plus 100 μ liter of GSSG (750 $m\mu$ mole). (D) Incubation mixture as in (C), dialyzed for 1 hour against 4 liters of 0.068 M phosphate buffer (*pH* 6.8) at 4 C plus 100 μ liter of GSSG (750 $m\mu$ mole) and 10 μ liter of NADPH (\pm 75 $m\mu$ mole) with GSSG reductase activity, 8 U. (E) 900 μ liter of 0.068 M phosphate buffer (*pH* 6.8), 100 μ liter of GSSG (750 $m\mu$ mole), 100 μ liter of CoASSG (51 $m\mu$ mole), 10 μ liter of diluted 1:10 purified yeast glutathione reductase (1 μ g of protein), 10 minutes of incubation, plus 10 μ liter of NADPH (\pm 75 $m\mu$ mole) with GSSG reductase activity, 108 U.

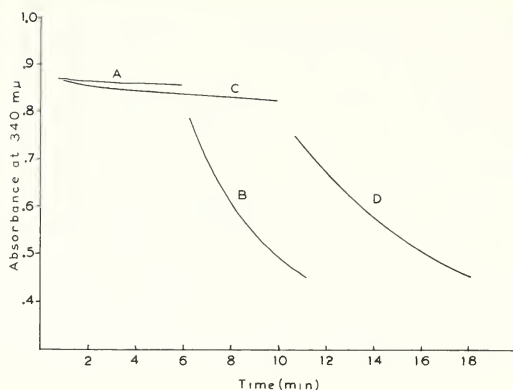
thermore, it has been possible to show that the presence of the hydrogen coenzyme donor is very important for the inhibition effect. If the coenzyme is avoided during the incubation period of the enzyme with the CoASSG (which in this case could act as an inhibitor), no inhibition can be demonstrated upon the addition of GSSG followed by NADPH (*curve E*).

Our studies on the inhibition effect by CoASSG have been extended to rat liver and yeast extracts prepared in our laboratory, which display both reducing activities. The inhibition effect by CoASSG is less accentuated in these extracts when a proportionally higher reducing activity (CoASSG reductase) is present (*see text-figs. 2, 3*).

In our laboratory the effect of 0.001 M EDTA on purified yeast glutathione reductase with CoASSG or GSSG as substrate was studied (table 1). Under this condition (EDTA) an appreciable breakdown of both the mixed disulfide and GSSG was noted within a 10-minute period. However, in the absence of EDTA, CoASSG, and GSSG reductase activity decreased.



TEXT-FIGURE 2.—Inhibition of laboratory prepared yeast GSSG-reductase (40–60% ammonium sulphate saturation) by a mixed disulfide complex (CoASSG). (A) 900 μ liter of 0.1 M phosphate buffer (pH 6.2), 20 μ liter of NADPH (± 100 m μ moles), and 40 μ liter of enzyme (5.5 mg of protein/ml). (B) Incubation mixture as in (A), after 4 minutes of incubation plus 40 μ liter of GSSG (300 m μ moles); GSSG-reductase activity, 0.043 U. (C) 900 μ liter of 0.1 M phosphate buffer (pH 6.2), 20 μ liter of NADPH (± 100 m μ moles), 40 μ liter of enzyme (5.5 mg or protein/ml); and 80 μ liter of CoASSG (102.8 m μ moles); CoASSG-reductase activity, 0.005 U. (D) Incubation mixture as in (C), after 10 minutes of incubation plus 40 μ liter of GSSG (300 m μ moles); GSSG-reductase activity, 0.015 U.



TEXT-FIGURE 3.—Inhibition of rat liver GSSG-reductase (40–60% ammonium sulphate saturation) by a mixed disulfide complex (CoASSG). (A) 900 μ liter of 0.068 M sodium phosphate buffer (pH 6.8), 20 μ liter of NADPH (± 130 m μ -moles), 5 μ liter enzyme (15.6 mg of protein/ml). (B) Incubation mixture as in (A) after 5 minutes of incubation plus 100 μ liter of GSSG (750 m μ -moles); GSSG-reductase activity, 0.198 U. (C) 900 μ liter of 0.068 M sodium phosphate buffer (pH 6.8), 20 μ liter of NADPH (± 130 m μ -moles), 5 μ liter of enzyme (15.6 mg of protein/ml) after 5 minutes of incubation, plus 20 μ liter of CoASSG (51 m μ -moles); CoASSG-reductase activity, 0.012 U. (D) Incubation mixture as in (C) after 10 minutes of incubation with CoASSG, plus 100 μ liter of GSSG (750 m μ -moles); GSSG-reductase activity, 0.105 U.

The enhancement of the two reducing activities, GSSG and CoASSG reductases, by EDTA in the purified yeast preparation cannot be easily explained, although this effect may be due to its chelating activity because an unexpected finding was that a rat liver extract, prepared in our laboratory, did not display a change in its normal activity on CoASSG when incubated with or without EDTA. With rat liver extracts the only enhanced activity by EDTA is GSSG reductase (table 1).

Recently Eriksson (8) has confirmed our finding of a CoASSG reductase activity in the purified yeast glutathione reductase preparation.

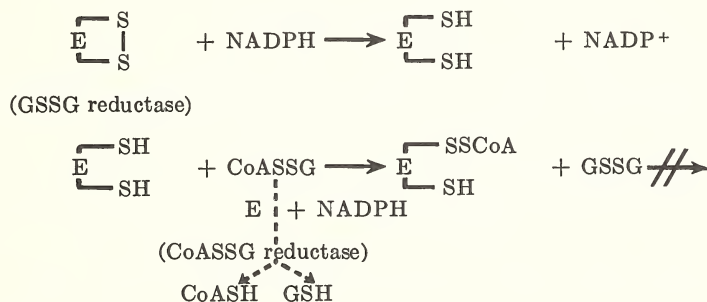
TABLE 1.—EDTA effect on purified yeast and rat liver CoASSG and GSSG reductases*

Source of enzyme	Type of activity	With EDTA (0.001 M)	Without EDTA
Purified yeast	CoASSG-reductase	2.1 U	0.2 U
	GSSG-reductase	81.0 U	14.0 U
Rat liver (40–60% ammonium sulphate saturation)	CoASSG-reductase	0.006 U	0.006 U
	GSSG-reductase	0.204 U	0.169 U

*All the results were obtained by measuring the initial velocities of the reactions with 50 μ M of CoASSG and 750 μ M of GSSG in sodium phosphate buffer (pH 6.8) at 22 C. One enzymic unit = 1 μ mole/minute/mg of protein. (The buffer was prepared with commercially distilled water.)

He reports 1.46 U in the presence of EDTA. Moreover, he mentions an inhibition by CoASSG of a glutathione reductase from porcine erythrocytes.

Fortunately, the reaction mechanism of yeast glutathione reductase has been carefully studied, and according to the recent results obtained by Massey and Williams (9), glutathione reductase has a protein disulfide active center in addition to the flavin. These authors report data which indicate that this enzyme contains a basically identical reaction mechanism to that of lipoyl dehydrogenase. Our finding regarding the inhibition of GSSG reductase with CoASSG in the presence of NADPH is in agreement with the observation by Massey and Williams that the NADPH reduces first an active center disulfide of the enzyme, permitting the formation of a protein-dithiol which in this case can interact with the mixed disulfide complex of CoASSG, forming a protein-SS-CoA complex, inactivating the enzyme activity for GSSG:



As to the nature of the new enzymic activity, one wonders if it belongs to the same protein molecule of GSSG reductase or whether it is a different enzyme. Although we have found some differences between these two activities with respect to NADPH and NADH dependency (4), heat lability, and optimum pH (10), up to now we have been unable to answer the above question, which seems of great interest, taking into consideration the possible regulation within the cell of GSSG reductase in the presence of mixed disulfide complex (CoASSG) that in turn can be broken down enzymically.

It is pertinent to mention that recently a CoASSG reductase from bovine kidney has been characterized by Chang and Wilken (11), but this seems to be a different enzyme from that studied in this laboratory with rat liver and yeast (4, 10).

RESUMEN

Existe una actividad reductora de CoASSG dependiente de NADPH en las preparaciones comerciales de reductasa de GSSG purificadas de levadura, así como en la reductasa de GSSG parcialmente purificada obtenida de levadura y de hígado de rata.

La actividad de reductasa de GSSG a partir de estas fuentes puede inhibirse por el complejo de disulfuro mixto (CoASSG) cuando la incubación se lleva a cabo en presencia de NADPH. El efecto inhibitor es parcialmente reversible por medio de diálisis. Si la enzima es incubada con su sustrato (GSSG) y CoASSG, en ausencia de NADPH, no puede demostrarse el efecto inhibitor. Debido a la degradación del CoASSG la inhibición es menos acentuada con los extractos parcialmente purificados de levadura y de hígado de rata.

El EDTA aumenta las actividades de reductasa de CoASSG y GSSG en la preparación de levadura, pero no muestra efecto alguno sobre la actividad reductora de CoASSG del hígado de rata.

Se discute el mecanismo de la inhibición de acuerdo con las observaciones de Massey y Williams de que el NADPH primero reduce un centro de disulfuro activo en la reductasa de GSSG que, en nuestro caso, permite la formación de un complejo proteína-SS-CoA.

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Oxygen Activation by *o*-Diphenols^{1,2,3}

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SUMMARY

o-Diphenols, including catecholamines, accelerate the autoxidation of *p*-hydroquinone, *p*-phenylenediamines and of *o*-diphenolic compounds themselves, probably by activating the oxygen molecule through charge transfer complexation. The possibility that the catalysis is exerted through products resulting from the autoxidation of the *o*-diphenol itself is shown to be untenable on several grounds. This *o*-diphenol catalysis has several impor-

tant effects: 1) It makes feasible other sequences of biological electron transport; 2) it explains the abnormally high O₂ uptake in a system of *p*-phenylenediamine and melanoma extracts, that is, the Greenstein-Riley phenomenon; 3) it may help in the understanding of the molecular biochemical role of natural *o*-diphenols, as well as the true importance of the enzyme polyphenol oxidase.—Nat Cancer Inst Monogr 27: 89–95, 1967.

THE ROLE of charge transfer complexation in accelerating biochemically important reactions is under study in our laboratories (1, 2). Especially interesting are autoxidation reactions, in view of the well-known peculiarity that entrance of one electron in oxygen will favor acceptance of a second electron. This may mean that oxygen is a better electron acceptor in the transition state than in the ground state; therefore, suitable donors may stabilize the transition state more than the ground state and thus speed up the reaction.

This paper reports catalysis of autoxidation reactions by charge transfer complexation of oxygen with catechol and catecholamines. This appears to be the first case of oxygen activation by species other than metallic ions.

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. H. A. Lardy, p. 125.

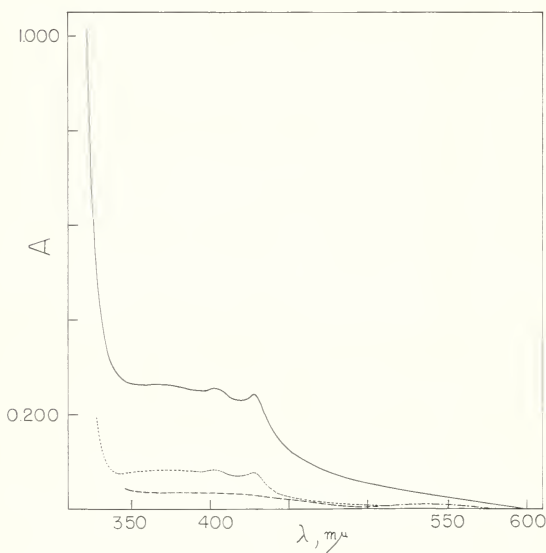
³ This work greatly benefited from grants of the Ford Foundation, the Brazilian "Conselho Nacional de Pesquisas," and the "Fundação de Amparo à Pesquisa" do Estado de São Paulo.

RESULTS

The activating effect of catechol and catecholamines has been studied in the autoxidation of 1) *p*-hydroquinone, 2) *p*-phenylenediamines, and 3) catechol itself, often by both spectrophotometric and manometric techniques.

Autoxidation of *p*-Hydroquinone

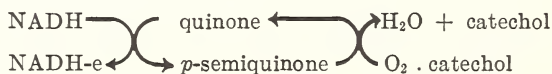
A fresh solution of *p*-hydroquinone shows some visible absorption, which is identical to that reported for the *p*-semiquinone. Catechol addition progressively enhances the *p*-semiquinone spectrum, quite rapidly if oxygen is bubbled through the solution (text-fig. 1); the excess absorption disappears to a considerable extent on acidification, thus confirming that it was due to a semiquinone.



TEXT-FIGURE 1.—Effect of 0.1 M catechol on the oxidation of 0.1 M hydroquinone. Spectra were taken after bubbling oxygen through the three solutions for 45 seconds. *Upper curve*, the mixture; *middle*, 0.1 M *p*-hydroquinone; *lower*, 0.1 M catechol. Solvent: 0.02 M phosphate buffer, pH 6.9, methanol (1:1, v/v).

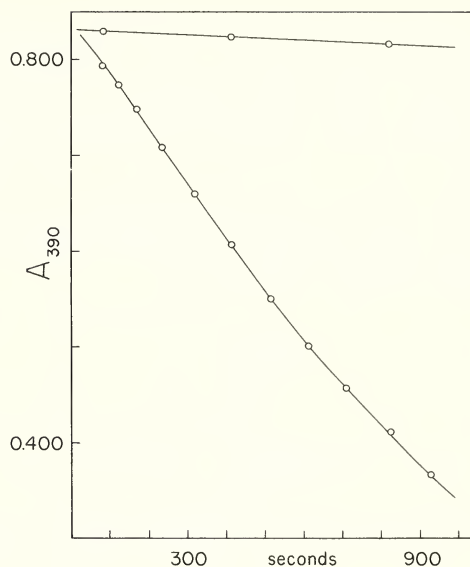
Reproduced from *Biochim Biophys Acta* 120 (1966) 84.

Since both *p*-semiquinone and *p*-quinone—the products of *p*-hydroquinone autoxidation—are unstable, it is more convenient to couple the catalyzed autoxidation with the oxidation of a dihydropyridine (text-fig. 2). In such a system the *p*-hydroquinone can be replaced by a quinone (text-fig. 3), even vitamin K₃. It appears that from the dihydropyridine and quinone a *p*-semiquinone is formed; this semiquinone, which can be spectrally detected, is then rapidly oxidized by catechol-activated oxygen:



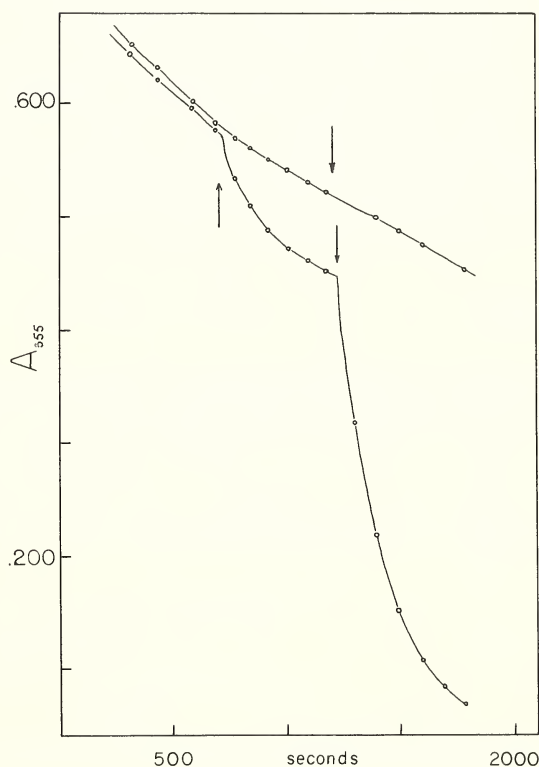
TEXT-FIGURE 2.—Combined effect of 2.85×10^{-2} M catechol and 2.85×10^{-2} M *p*-hydroquinone on the dehydrogenation of 3.48×10^{-4} M 1-benzyl-1,4-dihydronicotinamide in 0.02 M phosphate buffer, pH 6.4, methanol (1:1, v/v). *Upper curve* shows the effect of hydroquinone alone. The effect of catechol alone is even more negligible.

Reproduced from Biochim Biophys Acta 120 (1966) 84.



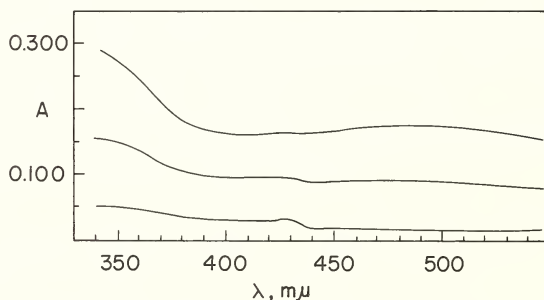
TEXT-FIGURE 3.—Effect of 2×10^{-2} M catechol and air on the dehydrogenation of 9.0×10^{-5} M 1-benzyl-1,4-dihydronicotinamide in the presence of 3.0×10^{-5} M chloranil in 0.02 M phosphate buffer, pH 6.9, methanol (1:1, v/v). Initially, the cells were carefully evacuated at 0 C. *Upper curve* indicates no catechol. *Upward arrow* indicates catechol addition; *downward arrows* indicate times air was admitted.

Reproduced from Biochim Biophys Acta 120 (1966) 84.



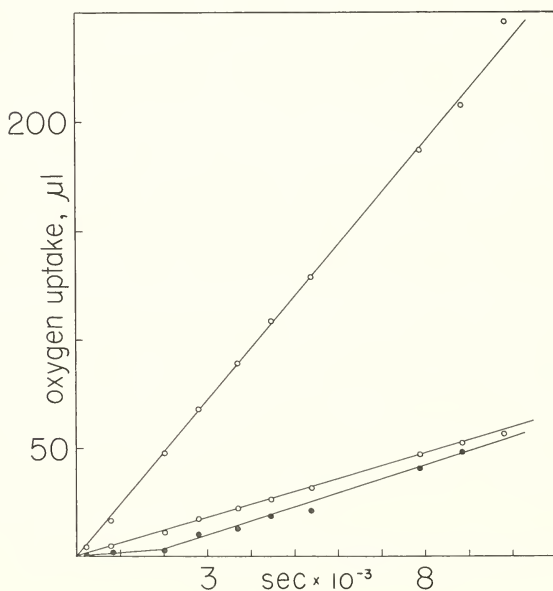
Catechol seems to be specific in its effect; the active entity appears to be the monoanion, which is effective in very low concentrations and does not need the cooperation of metal ions.

The first attempts to demonstrate spectrophotometrically that adrenal hormones speed up semiquinone formation from *p*-hydroquinone were inconclusive because of secondary reactions. Some success has now been achieved (text-fig. 4), but one can measure more easily the oxygen uptake and observe the abnormally high rate in the complete system (text-fig. 5).



TEXT-FIGURE 4.—Effect of 4.5×10^{-3} M nor-epinephrine on the autoxidation of 0.1 M *p*-hydroquinone in 0.1 M phosphate buffer, pH 7.2, mixed with methanol (1:1, v/v). The curves represent the spectrum of the mixture against that of the hydroquinone after 60 seconds (lower curve), 300 seconds (middle), and 570 seconds. The upper and middle curves have been displaced vertically by 0.1 and 0.05 in the absorbancy value, respectively. The hormone alone does not generate significant new absorption. Note the spectrum changes progressively from that of *p*-semiquinone to that of nor-adrenochrome.

Reproduced from *Biochim Biophys Acta* 143 (1967) 88 (Fig. 1).

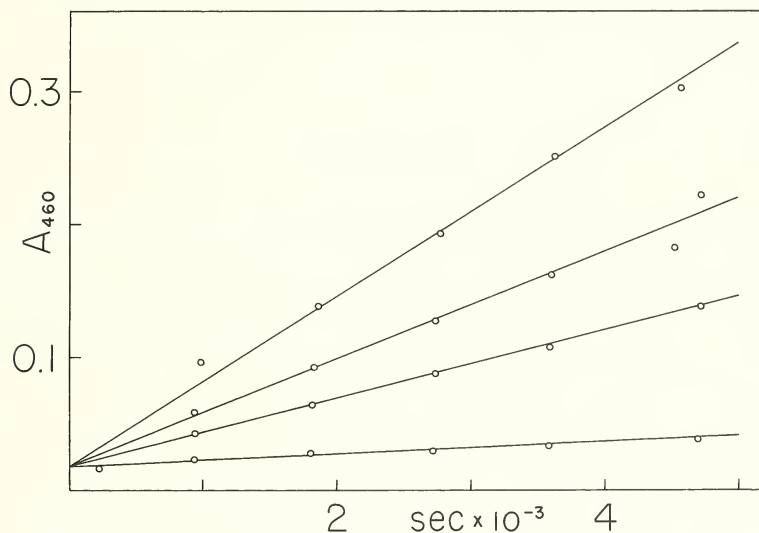


TEXT-FIGURE 5. — Increased oxygen uptake in the system of 1.1×10^{-2} M *p*-hydroquinone and 4.7×10^{-3} M epinephrine in 0.1 M phosphate buffer pH 7.50. Middle line, hydroquinone alone; lower line, epinephrine alone.

Reproduced from *Biochim Biophys Acta* 143 (1967) 88 (Fig. 3).

Autoxidation of *p*-Phenylenediamines

Since at neutral pH the semiquinone formed from the oxidation of *p*-phenylenediamines (3) is much more stable than that from *p*-hydroquinone, one can readily follow spectrophotometrically the catechol-catalyzed process (text-fig. 6).



TEXT-FIGURE 6.—Effect of catechol on the autoxidation of 3.0×10^{-3} M *p*-phenylenediamine as shown by the development of the semiquinone (radical cation) absorption maximum at 460 m μ . Solvent: 0.01 M phosphate buffer, pH 6.4, methanol (1:1, v/v). Catechol concentration (starting from the bottom): 0.0, 1.0, 2.5, and 5.1 mM.

Reproduced from *Biochim Biophys Acta* 143 (1967) 88 (Fig. 5).

Manometric data for the systems of *p*-phenylenediamine and catechol or DOPA are available from studies of the Greenstein-Riley effect (4), *i.e.*, the abnormally high oxygen uptake when melanoma extracts are tested with *p*-phenylenediamine.

Riley (4) found that the melanoma extracts can be substituted by DOPA and even by catechol, with melanin-like products being formed.

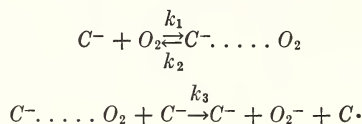
Autoxidation of Catechol

The rate of oxygen uptake is of the type (5)

$$\frac{B[C^-]^2O_2}{[C^-] + A},$$

where A and B are constants and $[C^-]$ represents the concentration

of the catechol monoanion. We obtained the above expression from the steady-state treatment of the following mechanism



in which one molecule of catechol is oxidized and another acts as activator, as required by our interpretation of *o*-diphenol catalysis of autoxidation.

DISCUSSION

The possibility that the catechol effect is exerted through products of its own autoxidation is ruled out on several grounds: 1) on a molar basis, catechol (5) consumes oxygen at a lower rate than *p*-hydroquinone (6), and it is therefore difficult to see how catechol could—by way of an oxidation product, even in a concentration 100 times lower—catalyze the oxidation of the *p*-isomer. Moreover, none of the manometric results reported can be explained by a cyclic role of an oxidation product. 2) Catechol semiquinone, had it been formed by air oxidation, would have oxidized the dihydropyridine (7). This would leave unexplained the necessity of *p*-hydroquinone or a quinone. 3) During a period of time comparable to that of the catalyzed dihydropyridine oxidation, a catechol control does not contain spectroscopically detectable products. 4) Above a certain concentration, the order of reaction with respect to catechol in the autoxidation of *p*-phenylenediamines may be zero.

It may well be that *in vivo* conditions for oxygen activation by *o*-diphenols are more favorable. In the case of catecholamines, if the amino group is fixed to a receptor, the formation of adrenochrome-type products is minimized. Alternatively, the formation of these products may be followed by the formation of 5,6-dihydroxy-indoles (8), and these should be very strong oxygen activators. It is very interesting that adrenochrome does indeed catalyze the autoxidation of epinephrine and of ascorbic acid (9).

Another inference from our work is that the enzyme polyphenol oxidase, which can form both the *o*-diphenol and the quinone, may have a key position in electron transport.

The enzymic formation of naphthosemiquinone radicals (10) acquires further significance as these radicals could shuttle electrons between reduced pyridine coenzymes and *o*-diphenol-activated oxygen.

Our work readily explains the Greenstein-Riley (4) phenomenon and related effects. Of these, the weaker toxicity of *p*-phenylenedia-

mine to melanoma-bearing mice compared to nontumorous controls may be due to increased metabolic oxidation of the drug by the catalytic effect of DOPA-like compounds present in great excess in the tumor.

RESUMEN

Los *o*-difenoles, incluyendo a las catecolaminas, aceleran la autooxidación de la *p*-hidroquinona, las *p*-fenilendiaminas y los propios compuestos *o*-difenílicos, probablemente activando la molécula de oxígeno a través de una formación de complejo dependiente de una transferencia de carga. Se ha demostrado por diversas razones que es insostenible la posibilidad de que la catálisis se ejerza por medio de productos resultantes de la autooxidación del propio *o*-difenol.

Esta catálisis por *o*-difenol tiene varios efectos importantes: 1) hace posible otras secuencias del transporte de electrones en biología; 2) explica las captaciones anormalmente elevadas de O₂ en un sistema de *p*-fenilendiamina y extractos de melanoma (es decir, el fenómeno de Greenstein-Riley); y 3) puede ayudar a comprender el papel bioquímico, a nivel molecular, de los *o*-difenoles naturales, así como su verdadera importancia para la enzima polifenol oxidasa.

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Regulatory Action of Ions on Some Mitochondrial Functions^{1,2}

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SUMMARY

The uptake of inorganic phosphate into adenosine triphosphate (ATP), the Ca^{++} -induced H^+ ejection, and the orthophosphate-induced mitochondrial swelling has been studied in rat liver mitochondria incubated in 0.4 M KCl or NaCl at pH. 6.0. It has been possible to demonstrate a potassium-sensitive incorporation of inorganic phosphate into ATP and in similar conditions, the Ca^{++} -induced H^+ ejection and the orthophosphate-induced mitochondrial swelling are also dependent on potassium ions. The results obtained in these studies provide evidence for the

existence of a potassium-sensitive reaction in mitochondria that is involved under certain conditions in ATP generation, but it is also probable that high-energy intermediates of oxidative phosphorylation can be utilized through this reaction to produce ejection of H^+ or structural changes in the mitochondria. The ultimate utilization of these high-energy intermediates through the potassium-sensitive reaction seems to depend on the ionic environment.—*Nat Cancer Inst Monogr* 27: 97-109, 1967.

SEVERAL YEARS AGO Pressman and Lardy reported that oxidative phosphorylation could become dependent on potassium ions (1). More recently, the effect of potassium ions on certain mitochondria functions has been studied; certain types of mitochondria swelling (2-6), as well as the ejection of H^+ , induced by valinomycin (3, 7), the synthetic glucocorticoid, triamcinolone,⁵ the surface active agent Tergitol

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. H. A. Lardy, p. 125.

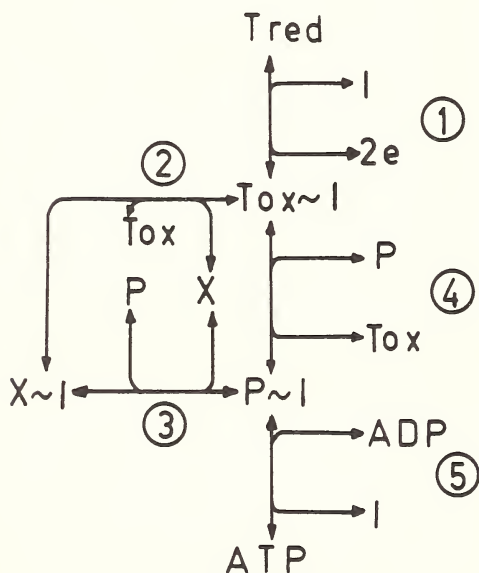
³ We are indebted to Dr. P. A. George Fortes for active collaboration in the swelling experiments, and to Mrs. Susana V. de Soria for excellent technical assistance.

⁴ Abbreviations: ADP and ATP, adenosine diphosphate and triphosphate respectively; DNP, 2,4-dinitrophenol.

⁵ The uncoupling action of triamcinolone is not due to this particular steroid, but to an impurity in some preparations. The latter substance has been isolated and is currently being synthesized.

NPX (8), and gramicidin (6) have been demonstrated to take place more effectively in the presence of potassium ions than in sodium ions.

In this respect, it has been argued by Pressman (3) that induced transport and mitochondrial swelling are processes that involve the utilization of chemical-bond energy for mechanical work such as active transport or structural changes, while Chappel and Crofts (6) have suggested that mitochondrial swelling is secondary to the active entrance of ions into the mitochondria. However, from studies made with the glucocorticoid, triamcinolone, in sodium media and in potassium media (9), it was postulated that there was a category of high-energy intermediates that was derived from the direct oxidative phosphorylation reaction through a potassium-sensitive reaction. The results obtained with the steroid were tentatively explained according to a scheme postulated in 1962 (10) (text-fig. 1) on the assumption that reaction 2 was potassium sensitive and that this reaction was somehow involved in the triamcinolone-induced mitochondrial swelling. The early work of Pressman and Lardy (1) might also support the hypothesis that mitochondria possess a potassium-sensitive reaction.



TEXT-FIGURE 1.—In this scheme, taken after Vignais *et al.* (10), T_{red} and T_{ox} is a respiratory carrier in its reduced or oxidized form, respectively. I and X are unknown substances in the mitochondria that can form high-energy intermediates during electron transport (e^-).

By incubation of mitochondria at high salt concentrations, it has been possible to show that liver mitochondria have a potassium-sensitive reaction linked in some manner to oxidative phosphorylation, and that this reaction may be manifested in various forms; it can be used to transfer chemical-bond energy, or to produce mitochondrial structural changes or active ejection of H^+ from the mitochondria.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to Schneider and Hogeboom (11) in 0.25 M sucrose. In all experiments the mitochondrial pellets were washed once with 0.25 M sucrose.

Incorporation of inorganic phosphate into ATP.—The reaction was studied in the incubating conditions indicated under "Results"; mitochondria from 100 mg of rat liver suspended in 0.1 ml of 0.25 M sucrose were used for the assay; after 15 minutes of incubation at 25 C, the reaction was stopped with trichloroacetic acid at 5% final concentration. After centrifugation an aliquot was taken and the incorporation of ^{32}P into ATP was measured according to Plaut (12). These determinations were checked by another series of experiments in which ATP was separated by paper chromatography and essentially the same pattern of results was obtained; this technique has been described before (13) and was used only to confirm the results obtained according to the method described by Plaut (12).

H^+ ejection.—Changes in pH in the indicated incubation mixture were followed with a Beckman expanded scale pH meter attached to a Beckman recorder.

Mitochondrial swelling.—The changes in optical density at 520 $\text{m}\mu$ were taken as a measure of mitochondrial swelling; a decrease was interpreted as swelling. In all experiments, the mitochondrial suspension in sucrose was adjusted so that 0.04 ml added to 3.0 ml of the indicated incubation mixture gave an initial optical density of 0.600–0.800.

Adrenosinetriphosphatase.—The activity of mitochondria from 200–250 mg of rat liver, suspended in 0.5 ml of sucrose, was assayed at 25 C for 15 minutes in the appropriate incubation mixture. The reaction was stopped by cold trichloroacetic acid at 5% final concentration. Inorganic phosphate was determined according to Sumner (14).

RESULTS

In agreement with work from other laboratories (15), mitochondria incubated in 0.4 M KCl in the absence of succinate incorporate orthophosphate into ATP at a very low rate. In the presence of succinate, a larger amount of inorganic phosphate is incorporated into ATP, especially when the incubation media contains potassium ions; in sodium media the uptake is not increased significantly (table 1).

These experiments are difficult to evaluate as to the extent to which the label in ATP is due to a ^{32}P -ATP exchange reaction as described by Boyer *et al.* (16, 17) and what amount of ^{32}P is incorporated into

TABLE 1.—Incorporation of inorganic phosphate into ATP*

Additions	μmole of inorganic phosphate incorporated into ATP	
	NaCl	KCl
None.....	82	179
Succinate.....	116	349
Malonate.....	80	117
Succinate + malonate.....	85	150

*Incubation mixture: 0.4 M KCl or NaCl, 0.01 M KH_2PO_4 or NaH_2PO_4 containing 10^6 cpm, 0.002 M Maleic Tris buffer (pH 6.0), ATP 11 μmole, 0.002 M succinate, 0.02 M malonate, 0.25 M sucrose, mitochondria from 100 mg of rat liver, final volume 1 ml, incubation time 15 minutes, temperature 25 C.

ATP in an energy-requiring process. In the all-sodium tubes, malonate in the absence of succinate does not diminish the uptake of ^{32}P into ATP, and the slight increase observed with succinate is completely inhibited by malonate (table 1). Apparently, malonate does not inhibit the exchange reaction, and the increased incorporation of inorganic phosphate into ATP observed in the presence of succinate is due to succinate oxidation. In potassium media relative to sodium media, a larger uptake is observed in all instances. Although malonate diminishes orthophosphate uptake in the absence of succinate, the inhibition is far more significant in the tubes containing succinate. Thus, it is valid to consider that the increased uptake of inorganic phosphate into ATP upon the addition of succinate is an energy-requiring process depending on succinate oxidation.

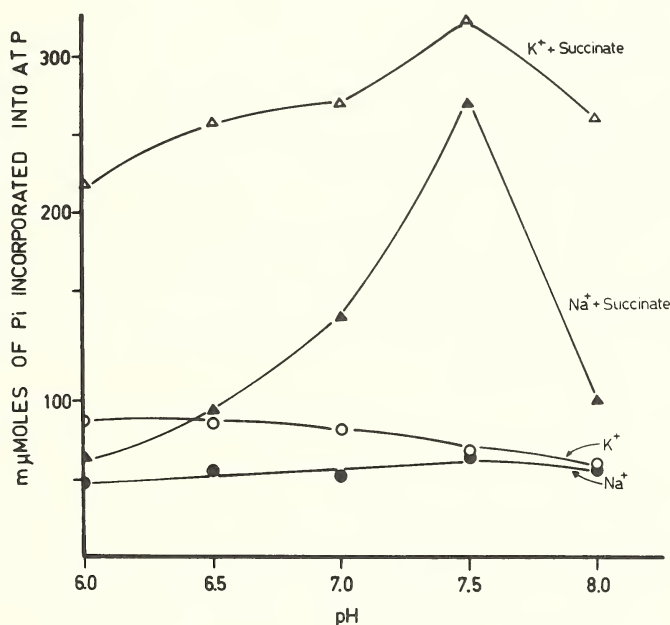
Further insight into the nature of this process is presented in table 2. ADP, as well as ATP, significantly stimulated orthophosphate uptake. Succinate in the absence of ADP or ATP does not increase the uptake. Antimycin A, which inhibits electron transport, as well as DNP and oligomycin significantly diminish the extent of the reaction.

TABLE 2.—Incorporation of inorganic phosphate into ATP*

Additions	μmole of inorganic phosphate incorporated into ATP
None.....	39
Succinate.....	127
ADP.....	53
Succinate + ADP.....	330
ATP.....	69
Succinate + ATP.....	210
Succinate + ATP + oligomycin (2 μg).....	49
Succinate + ATP + antimycin (2 μg).....	47
Succinate + ATP + DNP (10^{-4} M).....	43

*The incubating conditions were the same as those given in table 1; 3.0 μmoles of ADP were added where indicated.

The incorporation of ^{32}P into ATP, both in the presence and absence of succinate, was studied as a function of pH and gave the results presented in text-figure 2. In sodium media and in potassium media the uptake measured in the absence of succinate had no apparent maximum, but in the presence of succinate it resulted in an optimum pH around 7.5. In the all-sodium tubes, a significant pH optimum was found at 7.5; this value in sodium media almost reached the value obtained in all-potassium media; the highest K^+/Na^+ ratio was found in the 6.0–6.5 region.



TEXT-FIGURE 2.—Incorporation of inorganic phosphate into ATP in 0.4 M salt concentration as a function of pH . Experimental conditions: 0.4 M KCl or NaCl, 0.01 M KH_2PO_4 or NaH_2PO_4 containing 10% cpm (adjusted to the desired pH with Tris), 0.002 M Maleic-Tris buffer at the indicated pH , ATP disodium salt 11 μmole (adjusted to the indicated pH with Tris), 0.002 M succinate where indicated, 0.025 M sucrose, mitochondria from 100 mg of rat liver, final volume 1 ml, incubation time 15 minutes, temperature 25 C.

Ca^{++} -Induced H^+ Ejection

This phenomenon has been studied in rat liver mitochondria with the addition of Ca^{++} , but no significant differences were reported in the presence of potassium ions relative to sodium ions (18, 19), although important differences were detected in the rebound process. However,

upon the addition of Ca^{++} to mitochondria incubated at high salt concentrations and pH of 6.0, acid production becomes more important in potassium media than in sodium media (table 3). Antimycin A and DNP completely inhibit the phenomenon, while oligomycin is without effect.

TABLE 3.— Ca^{++} -induced H^+ ejection

Incubation mixture*	mμmole of H^+ ejected
Potassium media.....	216
Sodium media.....	180
Potassium media + antimycin.....	0
Potassium media + DNP.....	0
Potassium media + oligomycin.....	216

*The incubation mixture contained 0.4 M KCl or 0.4 M NaCl, 0.006 M Tris-HCl buffer, 0.002 M succinate (adjusted to pH 6.0 with either NaOH or KOH), 0.05 M sucrose in a volume of 5.0 ml. Where indicated 10 μg of antimycin A, 5 μg of oligomycin, or 10^{-4} M DNP were included in the incubation mixture. After an equilibration period of 2 minutes at room temperature, 650 mμmole of CaCl_2 were added. Acid production was measured 2 minutes after the addition of CaCl_2 . In this experiment each vessel contained 55 mg of protein. Final volume, 5.0 ml; room temperature.

The Ca^{++} -induced H^+ ejection thus depends on electron transport and on a high-energy intermediate that lies beyond the site of action of DNP and before locus of action of oligomycin. Similar results are obtained at pH 7.5. The presence of phosphate ions does not alter the pattern of the phenomenon.

The incorporation of inorganic phosphate into ATP and the Ca^{++} -induced H^+ ejection at high salt concentration would seem to share a common reaction and a common high-energy intermediate, as Ca^{++} strongly depresses inorganic phosphate uptake (table 4). The energy-dependent acid production by Ca^{++} apparently utilizes one of the intermediates involved in ATP generation through a common system in which a potassium-sensitive reaction is involved, as the two processes at pH 6.0 distinguish between potassium ions and sodium ions. However, the action of Ca^{++} is more unspecific for potassium ions as the K^+/Na^+ ratio of the Ca^{++} -induced H^+ ejection is much lower than the K^+/Na^+ ratio of the uptake of inorganic phosphate into ATP. Never-

TABLE 4.—Effect of calcium on the incorporation of inorganic phosphate into ATP*

Incubation mixture	mμmole of inorganic phosphate incorporated into ATP	
	—	+ Ca^{++}
ADP in potassium media.....	266	120
ADP in sodium media.....	119	85
ATP in potassium media.....	351	187
ATP in sodium media.....	60	63

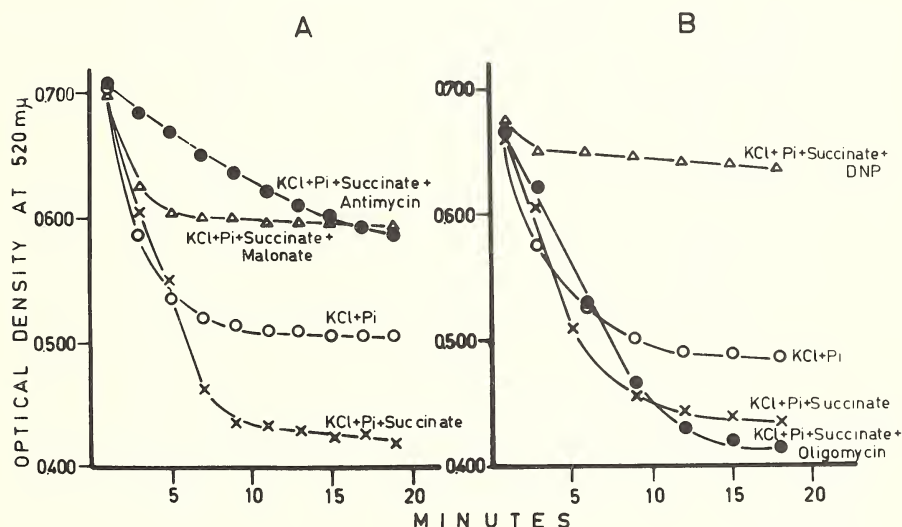
*The assays were done under the same conditions as given in table 2; all tubes contained 0.002 M succinate. 1.3×10^{-4} M CaCl_2 was added where indicated.

theless, in the presence of potassium ions and Ca^{++} , active incorporation of inorganic phosphate into ATP still occurs in a very important form although, simultaneously, a more significant acid production is taking place (tables 3 and 4). In the presence of potassium ions, the energy-dissipating reaction and the energy-retaining reaction work with a better efficiency.

Orthophosphate-Induced Mitochondrial Swelling

Potassium-dependent mitochondrial swelling as induced by certain agents such as valinomycin and gramicidin is a matter of current discussion, and it has been postulated that high-energy intermediates are utilized to produce structural changes in the mitochondria (3) and that mitochondrial swelling is secondary to the active entrance of potassium ions into the mitochondria (6). In both cases, uncoupling agents have been used to produce mitochondrial swelling, conditions in which energy expenditure would be expected. In this paper, a potassium-dependent mitochondrial swelling, with orthophosphate as the inducing agent, has been studied under conditions in which chemical-bond energy is not dissipated.

The extent of orthophosphate-induced mitochondrial swelling in potassium media is enhanced by the addition of succinate (text-fig. 3)

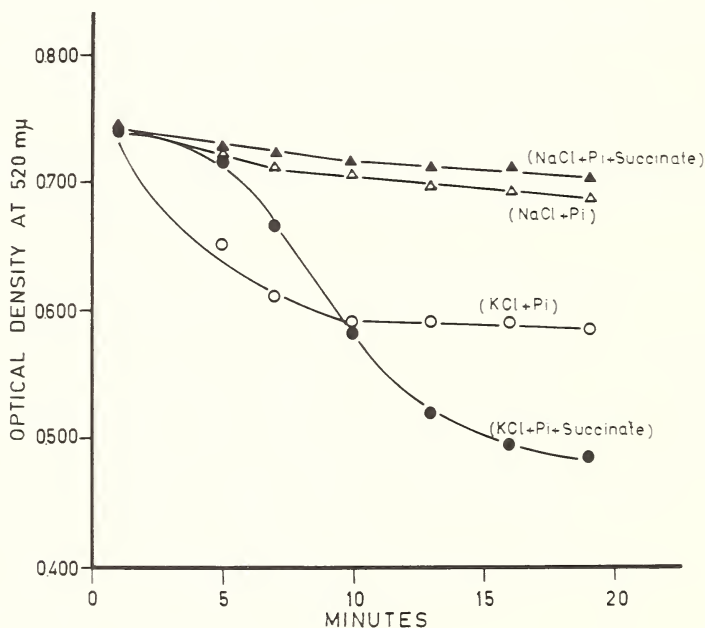


TEXT-FIGURE 3.—Effect of malonate, antimycin A, oligomycin, and DNP on the phosphate-induced mitochondrial swelling in the presence of succinate. Experimental conditions: in A and B all tubes contained 0.4 M KCl, 0.002 M Maleic-Tris buffer (pH 6.0), and 0.01 M KH_2PO_4 (pH 6.0), and, where indicated, 0.002 M succinate, 0.02 M malonate (adjusted to pH 6.0 with Tris), antimycin A (1 $\mu\text{g}/\text{ml}$), 10^{-4} M DNP, or oligomycin (1 $\mu\text{g}/\text{ml}$). Final volume 3.0 ml, room temperature.

or any other oxidizable substrate, although succinate in the absence of orthophosphate has no effect on mitochondrial swelling. Succinate-orthophosphate mitochondrial swelling is inhibited by malonate, antimycin A, and DNP, but not by oligomycin.

Mitochondrial swelling, in respect to its sensitivity to various inhibitors of electron transport or oxidative phosphorylation, is similar to the Ca^{++} -induced H^+ ejection except that in the acid production by Ca^{++} , as revealed by the inhibition of inorganic phosphate uptake into ATP, high-energy intermediates are dissipated, while in the swelling process bond energy is maintained.

The sensitivity of the swelling process to potassium ions is shown in text-figure 4. At pH 7.5, mitochondrial swelling takes place equally well in sodium or in potassium media and becomes independent of electron transport or high-energy intermediates.



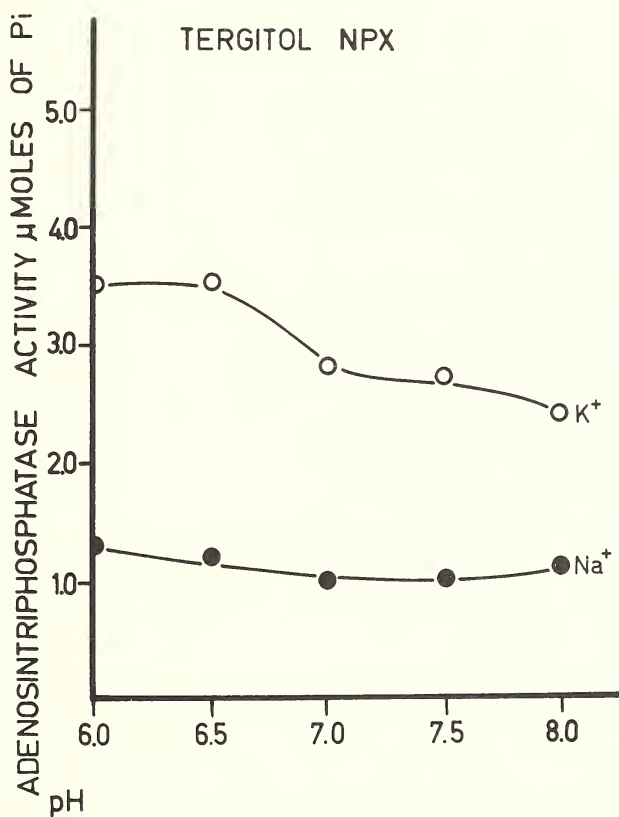
TEXT-FIGURE 4.—Mitochondrial swelling in potassium media and in sodium media. Experimental conditions: All tubes contained the indicated salt at 0.4 M, 0.002 M Maleic-Tris buffer (pH 6.0), 0.01 M KH_2PO_4 or NaH_2PO_4 (adjusted to pH 6.0 with Tris), and 0.002 M succinate where indicated. Final volume 3.0 ml, room temperature.

Mitochondrial Adenosinetriphosphatase Activity at High Salt Concentrations

Oxidative phosphorylation is generally considered a reversible process, but ATP in our experimental conditions failed to provide

high-energy intermediates which could be utilized to induce the Ca^{++} -induced H^+ ejection, or to increase the extent of mitochondrial swelling. Connelly and Lardy (20) by using a sucrose-histidine media have presented evidence that mitochondrial swelling induced by orthophosphate takes place to a maximum extent in the presence of ATP.

The reason for the discrepancy is unknown, but at 0.4 M KCl, oxidative phosphorylation also seems to behave as a reversible process. A latent adenosinetriphosphatase activity sensitive to potassium ions can be made apparent by low concentrations of Tergitol NPX (Union Carbide) (text-fig. 5). This activity is inhibited by oligomycin (table 5).



TEXT-FIGURE 5.—The stimulation of mitochondrial adenosinetriphosphatase activity by Tergitol NPX in 0.4 M salt as a function of $p\text{H}$. Experimental conditions: 0.4 M KCl or NaCl, ATP disodium salt 11 μmole (adjusted to the desired $p\text{H}$ with Tris), 0.02 M Maleic-Tris buffer at the indicated $p\text{H}$, 0.08 M sucrose, mitochondria from 200 mg of rat liver, final volume 1.5 ml, temperature 25 C. Tergitol NPX was added to 0.012% final concentration. Incubation time 15 minutes.

TABLE 5.—Effect of oligomycin on the Tergitol-NPX-stimulated adenosinetriphosphatase*

Additions	μ mole of Pi	
	– Oligomycin	+ Oligomycin
.....	1.2	0.6
Tergitol NPX.....	4.0	1.9

*Incubation mixture: 0.4 M KCl, ATP disodium salt (adjusted to pH 6.0 with Tris) 11 μ mole, 0.02 M Maleic-Tris pH 6.0, 0.08 M sucrose, mitochondria from 200 mg of rat liver, final volume 1.5 ml, temperature 25 C incubation time 15 minutes. Tergitol NPX was added to 0.012% final concentration.

DISCUSSION

The first evidence for the involvement of potassium ions in the formation of energized intermediates was reported by Pressman and Lardy (1), and it is probable that their results and the experiments described here are related. But, in view of the type of incubation system employed in the current studies, it is difficult to interpret the presently described results in terms of current concepts of oxidative phosphorylation.

Mitochondria have been incubated in 0.4 M KCl and under these conditions Lehninger *et al.* (15) reported inhibition of the 32 P-ATP exchange reaction and of the DNP-stimulated adenosinetriphosphatase activity. The ADP-ATP exchange reaction was not affected. These authors concluded that the reaction prior to the entrance of inorganic phosphate was blocked. This reaction on the Vignais *et al.* (10) scheme (text-fig. 1) corresponds to reaction 4. Nevertheless, other effects of high concentrations of KCl or NaCl are worth noticing; oxygen uptake by mitochondria incubated at high salt concentrations is not increased by ADP nor by DNP and thus, for instance, a diminishing action of high salt concentrations on electron transport cannot be discarded. On the considerations of Lehninger *et al.* (15), it has been assumed that reaction 4 of the scheme (text-fig. 1) is blocked by high salt concentrations (DNP does not stimulate adenosinetriphosphatase activity at 0.4 M KCl and pH 6.0).

Under these conditions all reactions involved in the formation of high-energy intermediates that subsequently lead to the generation of ATP must be made by a set of reactions that do not lie on the pathway of reaction 4. These reactions have been outlined as reactions 2 and 3 in text-figure 1. The potassium-sensitive incorporation of inorganic phosphate into ATP must be made through these two reactions. Furthermore, the sensitivity of the system to potassium ions may be localized, in agreement with previous work (9), to reaction 2 of the

scheme according to the following observations. Orthophosphate-induced mitochondrial swelling and the Ca^{++} -induced H^+ ejection, to a lesser extent, are potassium dependent. Since these two electron-transport requiring systems are inhibited by DNP, but not by oligomycin, which at high salt concentrations also inhibits phosphate transfer (tables 2 and 4), it can be postulated that the potassium-sensitive reaction would correspond to reaction 2 of the scheme.

This particular reaction would seem to possess certain interesting characteristics. Through reaction 2, inorganic phosphate promotes structural changes in the mitochondria by a potassium-sensitive mechanism; either reaction 2 by itself or the formation of $\text{X} \sim \text{I}$ would favor mitochondrial swelling. The present experiments substantiate the conclusions of Connelly and Lardy (20) and of Gómez-Puyou *et al.* (9), who implied that high-energy intermediates that do not lie on the route of direct oxidative phosphorylation are involved in certain types of mitochondrial swelling.

Mitochondrial swelling is an energy-preserving reaction. This conclusion does not agree with that of Pressman (3) nor with that of Chappell and Crofts (6), since both authors claim that induced potassium-dependent mitochondrial swelling is an energy-dissipating reaction. However, it should be noted, both groups have used uncoupling agents to induce the phenomenon, which differentiates their study from the present work in which uncouplers have been avoided.

The potassium-sensitive reaction is also involved in the Ca^{++} -induced ejection of H^+ in an energy-dissipating phenomenon. Since Ca^{++} clearly inhibits the uptake of inorganic phosphate, the H^+ ejection probably takes place by an unspecific hydrolysis of a high-energy intermediate with subsequent acidification of the media, as suggested by Chance (21). If both $\text{T}_{\text{ox}} \sim \text{I}$ and $\text{X} \sim \text{I}$ were hydrolyzed by the action of Ca^{++} , the low K^+/Na^+ of the Ca^{++} -induced H^+ ejection could be explained, as the formation of $\text{T}_{\text{ox}} \sim \text{I}$ is independent of potassium ions.

From the data in the present paper, it can be assumed that the final course of this reaction would depend on the ionic environment. In the presence of Ca^{++} , acidification of the medium results; in the presence of inorganic phosphate without phosphate acceptors, structural changes take place. The latter process, if supplemented with a suitable phosphate acceptor, results in ATP formation. The sensitivity of the reaction to a particular cation (potassium in this case) depends on the hydrogen-ion concentration.

RESUMEN

Se han estudiado la captación de fosfato inorgánico por el ADP para formar ATP, la salida de H^+ provocada por Ca^{++} , y la hinchazón mitocondrial provocada por

ortofosfato, en preparaciones de mitocondrias de hígado de rata incubadas en KCl o NaCl 0.4 M a pH 6.0.

Se ha podido demostrar una incorporación de fosfato inorgánico en el ATP, sensible al potasio; en condiciones semejantes, la salida de H^+ provocada por Ca^{++} y la hinchazón mitocondrial provocada por ortofosfato también dependen de los iones de potasio.

Los resultados obtenidos en estos estudios aportan pruebas de la existencia de una reacción sensible al potasio en las mitocondrias, que, en ciertas condiciones participa en la generación de ATP; sin embargo, también es probable que los intermediarios de alta energía de la fosforilación oxidativa puedan utilizarse por medio de esta reacción, para provocar la salida de H^+ o los cambios estructurales en las mitocondrias. La utilización última de estos intermediarios de alta energía, a través de la reacción sensible al potasio, parece depender de la atmósfera iónica.

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Lactic Dehydrogenase Activity and Aerobic Glycolysis in Tumors^{1,2}

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SUMMARY

It has been postulated that tumor tissue shows a high glycolytic rate because of a low activity of glycerol phosphate dehydrogenase and β -hydroxybutyric dehydrogenase; at the same time an elevation of lactic dehydrogenase (LDH) activity is observed. Accordingly, the specific inhibition of LDH could open a new possibility toward inhibition of tumor growth. The compound *N*-(4-carboxy-2-hydroxyphenyl)-maleimide was designed and synthesized, after Baker's ideas of "nonclassic antimetabolites." This compound acts as an irreversible inhibitor of LDH showing a K_i of 1×10^{-4} moles \times $1t^{-1}$. Oxamate (a competitive inhibitor of LDH) protected the enzyme against such irreversible inactivation, which points to an inhibition at the active site. When maleimide-inhibited LDH was dialyzed, no recovery of the activity

was obtained, but when oxamate-inhibited LDH was dialyzed, a full reactivation was obtained. Tumor tissue shows an elevation of LDH M isozyme, and a specific inhibitor of this isozyme might possess antitumoral activity. Maleimide is such an inhibitor. Biological assay showed the following results: No activity was found against ascites tumor and Sarcoma 180. On the other hand, in Sprague-Dawley rats bearing mammary carcinoma induced with 7,12-dimethylbenz[*a*]anthracene, maleimide significantly inhibited the appearance and development of the tumor. This inhibition of LDH can be used profitably for some studies dealing with its participation in the regulation of carbohydrate metabolism.—*Nat Cancer Inst Monogr* 27: 111–124, 1967.

A FUNDAMENTAL observation concerning the biochemistry of tumors was made by Warburg (1), who showed that these tissues are characterized by very elevated levels of both anaerobic and aerobic

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. H. A. Lardy, p. 125.

glycolysis. The second is especially distinctive (2), since except for certain normal tissues such as the retina (3, 4), the mucous membrane jejunum, kidney medulla (5), and myeloid tissues (6), other tissues do not form appreciable quantities of lactic acid in the presence of oxygen. Rees and Huggins (7) showed that tumors possess a very high lactic dehydrogenase (LDH) activity, and Huggins (8) considered that the capacity to synthesize appreciable quantities of this enzyme is a definitive characteristic of cancerous tissue.

Boxer and Devlin (9) recently proposed a plausible explanation for this phenomenon. Several of the tumorous tissues they studied lacked, or possessed very small quantities of, the enzymes glycerol-3-phosphate dehydrogenase (GPD) and β -hydroxybutyrate dehydrogenase (HBD). They therefore suggested it was to be expected that biochemical alterations such as the increase of aerobic glycolysis and of LDH synthesis would be produced.

During glycolysis, there is only one oxidation reaction, the conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate, with the participation of the corresponding dehydrogenase and the coenzyme nicotinamide adenine dinucleotide (NAD^+). In this reaction, two molecules of NADH are generated, which must be reoxidized for continuation of glycolysis. It is known that the multienzyme complex which catalyzes glycolysis is found in the "soluble fraction," or cytoplasm, of the cell (10, 11), and since Lehninger (12) has demonstrated that whole mitochondria are incapable of oxidizing external NADH in the presence of oxygen, the NADH formed during glycolysis must also be reoxidized in the cytoplasm. Normally, this oxidation takes place principally via GPD and HBD, whose substrates are dihydroxyacetone-phosphate and acetoacetate; the products, α -glycerophosphate and β -hydroxybutyrate, are able to penetrate the mitochondrion, and undergo oxidative degradations there. This explains why normal tissues do not form appreciable quantities of lactic acid: The cytoplasmic NADH is oxidized as described, and pyruvate is not reduced to lactate to any appreciable extent (except in muscular tissue). But tumorous tissue lacks the enzymes GPD and HBD, and the only pathway known at present for the reoxidation of cytoplasmic NADH is via LDH. The re-formed NAD^+ can then be used for the oxidation of more glucose, providing the large amounts of energy needed for the characteristically rapid growth of the tumor.

It is likely, therefore, that selective inhibition of LDH might be chemotherapeutic for cancer (9, 13-15). Although LDH is found in almost all tissues, its function (with the exception of skeletal muscular tissue) seems minimal, since the ubiquitous GPD and BHD provide the usual means of reoxidation of NADH produced during glycolysis.

It is now known that there are two principal forms of LDH, LDH_1 or M, and LDH_5 or H (16-21); respectively, these are the principal

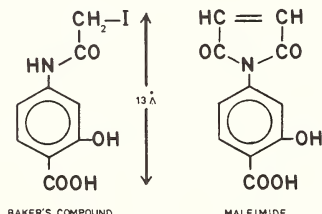
forms found in skeletal muscle and myocardial muscle. The pattern of the five isoenzymes which can be shown in almost all tissues (22-25) corresponds to the five possible combinations of four of the single subunits present in the above two forms: LDH₁ (HHHH), LDH₂ (HHHM), LDH₃ (HHMM), LDH₄ (HMMM), and LDH₅ (MMMM). The isoenzymes M and H have the same molecular weight (135,000), and similar physiochemical characteristics, except for isoelectric point and electrophoretic mobility (25). The latter differences allow their separation by starch gel electrophoresis and by DEAE-cellulose chromatography where LDH₁ only is retained (26). Enzymically, the isoenzyme H is inhibited by concentrations of pyruvate much smaller than those necessary to inhibit M (25). The amino acid composition of the two isoenzymes is different (25, 27-29), and so are their "fingerprints" (30, 31), indicating that their primary structures are different. "Fingerprint" differences are also found between the LDH's of different animal species, such as the rabbit, pig, and chicken (32). An important fact in relationship to the present work is that the predominant isoenzyme in tumors has been shown to be M (33). Theoretically it should be possible to selectively inhibit this particular isoenzyme. It is worthwhile stressing that preferentially anaerobic tissues (such as most tumors) possess isoenzyme M, whereas preferentially aerobic tissues (such as myocardial tissue) possess isoenzyme H, and would remain unaffected by this treatment.

That this rational approach to cancer chemotherapy might be useful was demonstrated in the studies of Papaconstantinou and Colowick (13, 34), who showed that oxamate, a competitive inhibitor of LDH (15), inhibited the growth of tissue cultures and of experimental tumors in animals.

The inhibitors studied in the present work were designed with the features proposed by Baker (35, 36), and Baker *et al.* (37, 38). They belong to the new class of "nonclassical antimetabolites," or "active-site-directed irreversible inhibitors" (39). These nonclassical antimetabolites differ from the classical in having a substantially increased size compared with the metabolite to be antagonized. The functional groups necessary for the specific attachment of the inhibitor to the enzyme are retained, and, in addition, a reactive group capable of reacting with the enzyme protein is included in the bulky groups that increase the size of the molecule. This type of inhibitor therefore acts irreversibly.

Baker, Lee, and Tong (38) have presented evidence that 4-(iodoacetamido)-salicylic acid inactivates LDH and glutamic dehydrogenase (GDH) by an irreversible inhibition directed against the active site. Baker arrived at the conclusion that his compound reacted preferentially with an amino group in GDH, and possibly also in LDH, although more recently it appears more likely that an -SH group is

involved (39). Assuming this, we tried to design an irreversible inhibitor directed against the active site by use of a group more specific for -SH than iodoacetate, which can also combine with other nucleophilic groups such as OH, NH₂, COOH, etc. Accordingly, the compound *N*-(4-carboxy-2-hydroxyphenyl)maleimide (maleimide) was synthesized. The structures of Baker's compound and maleimide are shown in text-figure 1.



TEXT-FIGURE 1.—Structures of Baker's compound and the *N*-(4-carboxy-2-hydroxyphenyl) maleimide.

This compound was tested for its selective and irreversible action against LDH with the further intention of study of its possible anti-tumor effect.

MATERIALS AND METHODS

Baker's inhibitor and the one mentioned above were characterized by elemental analysis, infrared and NMR spectroscopy, and equivalent weight determination by titration. Baker's compound was prepared by the method of Van der Stelt, Voorspuij, and Nauta (40). LDH was from rabbit muscle (twice crystallized, Calbiochem, Los Angeles, Calif.). NADH and pyruvate were from Sigma, and 7,12-dimethylbenz[*a*]anthracene from Eastman. The isoenzymes M and H were obtained from C. F. Boehringer and Soehne, Mannheim.

Procedure for demonstrating the inhibition of LDH.—The method of Baker, Lee, and Tong (38) was used. To three flasks, numbered 1 through 3, were added 20, 17, and 17 ml of 0.05 M Tris, pH 7.4. In flask number 4, we prepared the enzyme solution by mixing 17 ml of 1.4 mM NADH, 10 ml of Tris, and 3 ml of LDH solution (prepared by dissolving 0.1 ml of the original suspension in 9.9 ml Tris). We rapidly checked the LDH activity by mixing 1 ml of the enzyme-NADH solution with 2 ml of Tris in a quartz cuvette with a 1 cm light path. After reading the absorbancy at 340 m μ in a Zeiss PMQ-II spectrophotometer, we added 0.1 ml of 31 mM sodium pyruvate, and the change of absorbancy was read after 1 minute. This should be 1.0 or greater; if not, more LDH was added. In any case, 3 ml more of Tris was added to flask 4 to increase the volume to 32 ml.

Three milliliters of a 10.6 mM solution of Baker's inhibitor and 3.0 ml of 10.6 mM maleimide were added to flasks 2 and 3, respectively. Flask 1 served as a control without inhibitor. All four flasks were preincubated at 37 C for 5 minutes, after which 10 ml of enzyme-NADH solution was added to each of the first three flasks. At appropriate time intervals 3.0 ml aliquots were transferred to tubes, which were then placed in an ice bath to prevent further enzyme inactivation. Later, these tubes were warmed to 25 C, emptied into cuvettes, and the changes in absorbance at 340 m μ , produced in 1 minute on addition of 0.1 ml of 31 mM pyruvate, were measured.

Demonstration of irreversible inhibition by dialysis.—The procedure was similar to that described above. Samples from the flasks 1 through 3 were taken at 10, 30, and 60 minutes, and the remaining solution was tied in cellophane dialysis tubing which had been washed for 24 hours with Tris. Dialysis was performed at 4 C against 400 ml 0.05 M Tris, pH 7.4, with magnetic stirring. The Tris was changed every 12 hours, and dialysis was considered complete when no more NADH was found in the dialysate. After addition of NADH, to replace that lost during the dialysis, the LDH activity was measured as described.

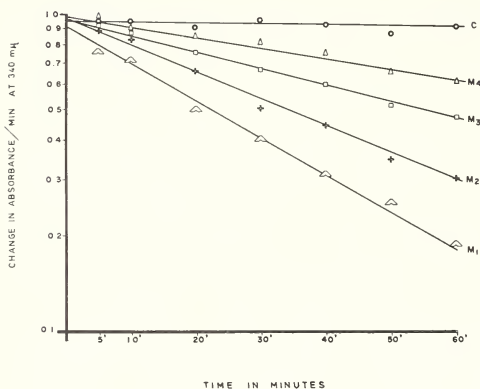
Induction of mammary carcinoma in rats.—The procedure of Huggins (8, 41) was followed. A single dose of 7,12-dimethylbenz[a]anthracene (20 mg) dissolved in 1 ml of sesame oil was administered to female Sprague-Dawley rats, 50–65 days old. With this critical combination of strain, sex, and age, 100% induction of mammary carcinoma is found between 28 and 92 days after the injection.

In these experiments, 125 rats, 51 days old, were used; 90 survived. Fifty-one days after the injection, 28 rats showed a tumor. At this point the rats were randomly divided into two groups: one with 47 rats (34 still without a tumor and 13 with) to serve as controls, and the other with 43 rats (28 still without a tumor and 15 with) for treatment with maleimide. The maleimide was administered in 0.5 ml saline, a dose of 32 mg/kg weight being used. Rats in the control group were given daily intraperitoneal injections of 0.5 ml saline, except where otherwise indicated.

RESULTS

Text-figure 2 shows the effect of four different concentrations of maleimide on LDH. The same solution of LDH-NADH was used in each determination.

The enzymic activity is plotted on a logarithmic scale because at a ratio of inhibitor enzyme of 10^6 the irreversible inhibition reaction is first order with respect to enzyme, and zero order with respect to inhibitor. Therefore the relative effects of two inhibitor concentrations



TEXT-FIGURE 2.—Effect of maleimide on LDH activity. ○—C, control; △ M₁, 3×10^{-4} M maleimide; + M₂, 2×10^{-4} M; □ M₃, 1×10^{-4} M; △ M₄, 5×10^{-5} M.

at any time is given by the ratio of the slopes of the lines at this time, after corrections have been made for spontaneous enzyme inactivation.

When the concentration of maleimide was increased from 1 to 2×10^{-4} M, the velocity of inactivation increased only 1.32 times. If the maleimide had reacted with the LDH by bimolecular collision (*i.e.*, tail alkylation), the velocity would have been doubled on doubling the inhibitor concentration. If, however, the maleimide reacted by exo-alkylation inside the enzyme-inhibitor complex, the velocity of inactivation would depend on the concentration of the complex. Assuming that the complex is in equilibrium with the components, it is clear that, on doubling the inhibitor concentration, the quantity of the complex would not be doubled owing to displacement of the equilibrium to a value between 1 and 2, depending on the magnitude of the dissociation constant K_i (34b) (38).

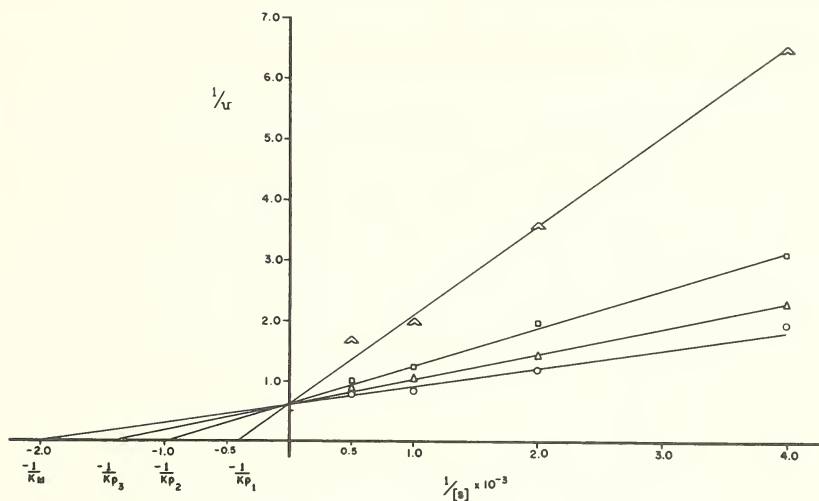
We made determinations of K_p (K_m modified by the inhibitor) by incubating the mixture of LDH-NADH with three different inhibitor concentrations for 10 minutes and then taking aliquots and measuring the enzyme activity by use of four different substrate concentrations. The results are shown in text-figure 3.

These values of K_p were plotted against inhibitor concentration (text-fig. 4). The point of intersection on the ordinate (42) gave a value of 1×10^{-4} for K_i .

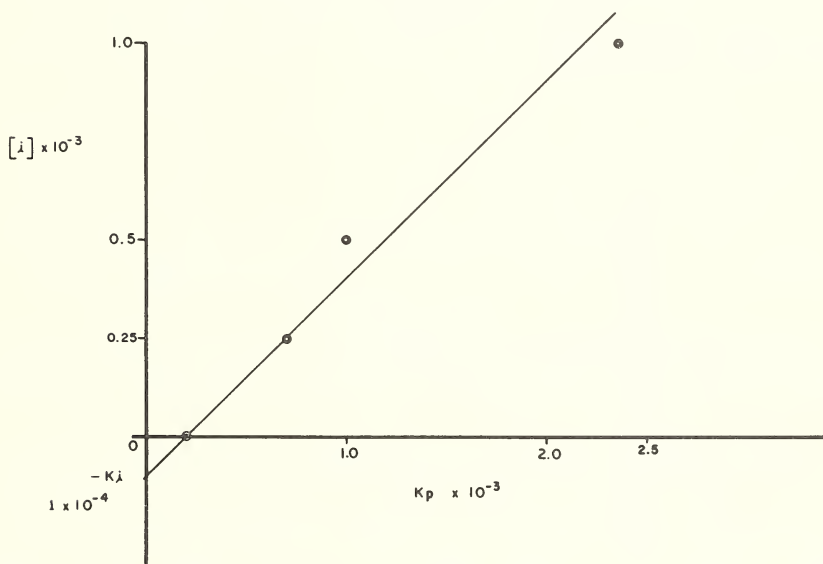
Protection by Oxamate Against the Irreversible Inhibition Produced by Maleimide

Text-figure 5 shows the effect of 0.3 mM sodium oxamate on the velocity of inactivation of LDH-NADH by 1×10^{-3} M maleimide. The ratio of the slopes shows a decrease of 1.23 times.

The protection by a competitive reversible inhibitor against inactivation of an enzyme by an irreversible inhibitor is considered to be decisive evidence that the effect of the irreversible inhibitor is on



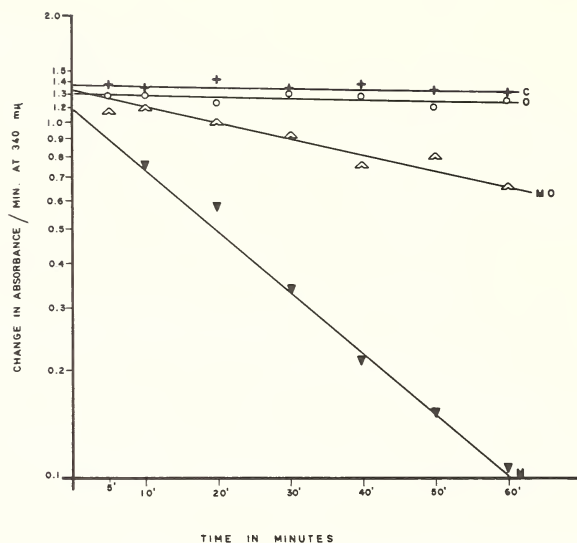
TEXT-FIGURE 3.—Determination of K_p for LDH: \circ , LDH-NADH control; \wedge , with 1×10^{-3} M maleimide; \square , 5×10^{-4} M; \triangle , 2.5×10^{-4} M.



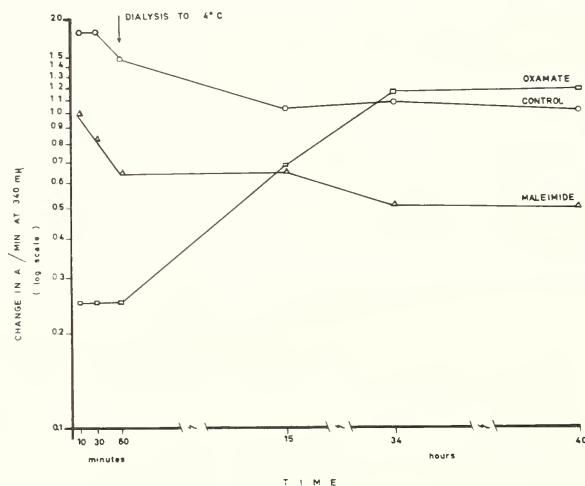
TEXT-FIGURE 4.—Determination of K_i for the reaction of LDH with maleimide. Values obtained from text-figure 3.

the active site (43). The reversible nature of the inhibition of LDH produced by oxamate has previously been demonstrated (13, 15, 34).

The protection which 0.3 M oxamate would afford is calculated after Baker's method (37) to be 1.27. If, on the other hand, maleimide inter-



TEXT-FIGURE 5.—Protection by oxamate against inactivation of LDH by maleimide. C (+) control; O, O (o) oxamate (0.3 mM) + LDH-NADH; M (▼), maleimide (10^{-3} M) + LDH-NADH; MO (△), maleimide (10^{-3} M) + oxamate (0.3 mM) + LDH-NADH.



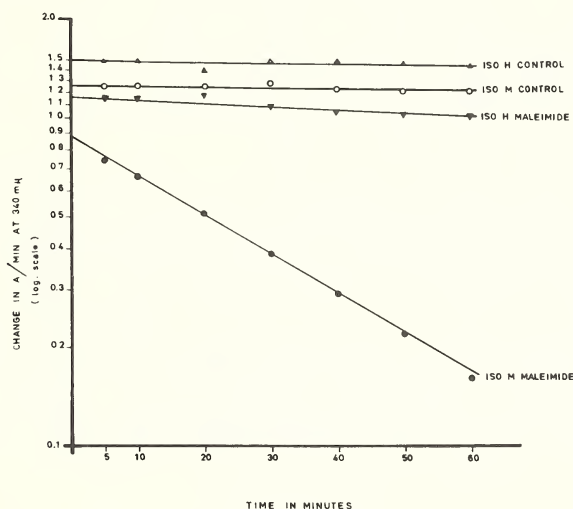
TEXT-FIGURE 6.—Demonstration of irreversible nature of maleimide inhibition. Following exposure of LDH to 1 mM maleimide or 3 mM oxamate (in presence of NADH) the samples were dialyzed to remove inhibitor and assayed for residual LDH activity.

acted with LDH by "tail alkylation," the inactivation should be decreased by a factor of 4.

Text-figure 6 shows the results from a dialysis experiment that demonstrated the irreversible inhibition of LDH maleimide and reversible inhibition by oxamate.

Effect of Maleimide on the Isoenzymes M and H of LDH

These enzymes were derived from rabbit muscle (M) and pig heart muscle (H) and were shown to be electrophoretically pure on polyacrylamide gel electrophoresis (44). Text-figure 7 shows the effect of maleimide on the isoenzymes.



TEXT-FIGURE 7.—The effect of maleimide on the pure M and H isoenzymes: ▲, control, H; ○, control, M; ▼, H + 10^{-3} M maleimide; ●, M + 10^{-3} M maleimide.

Effect of Maleimide on Induced Mammary Carcinoma in Rats

These results are given in table 1. Of the 15 rats with palpable tumors in the treated group, 9 had tumors of less than 1 cm diameter and 6 had tumors of 1–3 cm diameter. Of the 13 rats with tumors in the control group, 8 had tumors less than 1 cm in diameter, 4 had tumors of 1–3 cm diameter, and one had a tumor greater than 3 cm in diameter. The χ^2 test showed that both groups were homogeneous.

At the end of the treatment, the results were as follows: a) In the treated group, 14 rats did not develop a tumor (or there was regression in some), as against the control group, in which all rats developed tumors; b) 13 rats in the treated group showed tumors of less than

TABLE 1.—Relative size of mammary carcinoma from Sprague-Dawley rats during the treatment*

Size of tumors (cm)	Number of animals	At beginning of treatment (51 days after induction)	At 32 days of treatment	At 54 days of treatment	Number of animals	At end of experiment
Treated	43				43	
0		28	14	18		14
<1		9	26	17		13
1-3		6	15	19		29
>3		0	1	0		16
Controls	47				47	
0		34	15	14		0
<1		8	26	20		14
1-3		4	16	22		41
>3		1	3	12		59
χ^2		—	—	11.6		32.5
<i>P</i>		NS	NS	0.01		0.001

*Date of induction: May 6, 1964. Daily treatment from July 2 to September 4, with 32 mg maleimide/kg by intraperitoneal injection. The control animals were given injections of saline. Break in the treatment from September 4 to October 4. Treatment on every other day from October 4 to December 4. No further treatment was given from December 4 to February 6, 1965, and on this date the animals were killed.

1 cm diameter, against 14 in the control group; c) 29 rats in the treated group showed tumors of 1-3 cm in diameter, against 41 in the control group; d) 16 rats in the treated group showed tumors of greater than 3 cm in diameter, against 59 in the controls. The χ^2 test gave a value of $P < 0.001$, indicating that this difference is highly significant.

DISCUSSION

The inhibition of LDH by *N'*-(4-carboxy-2-hydroxyphenyl) maleimide has been characterized as competitive, as defined by Lineweaver-Burk analysis. Furthermore, the inhibition is irreversible since there was no recovery of LDH activity upon dialyzing the enzyme-inhibitor complex. This is in contrast to the case of another competitive inhibitor, oxamate, where the inhibition was lost on removal of the inhibitor. The reaction of both inhibitors, maleimide and oxamate, with the same site on the enzyme is demonstrated further by the observation that the irreversible inhibition by maleimide is prevented for the most part by the presence of oxamate (43). Thus these two competitive inhibitors most probably are effective by competing with pyruvate for the substrate site of the enzyme, but the maleimide, in contrast to oxamate, forms additional and stronger bonds with the enzyme that prevent its removal on dialysis.

The fact that isoenzyme H is not inhibited by maleimide, whereas isoenzyme M is (text-fig. 7), suggests that one of the differences between the isoenzymes is the distance between the -SH group and the active site necessary for substrate attachment. This must be 13 Å in isoenzyme M, but different in H, as judged from the dimensions of maleimide measured on scaled molecular models. Baker's compound and 4-maleamyl-salicylic acid only inhibit LDH derived from skeletal muscle and not that of myocardial muscle, so that they would probably act in a way similar to maleimide on the pure isoenzymes. In any case, maleimide is more active than either of these compounds.

Knowing that isoenzyme M is predominant in tumors (33), it would be expected that an inhibitor selective for this isoenzyme would act preferentially against this tissue, thus blocking the principal pathway of reoxidation of NADH liberated by glycolysis.

Maleimide might also be used for the determination of the levels of the isoenzymes M and H in various organs, a possibility which we are at present studying. Moreover, it might be used to investigate the importance of LDH in the metabolism of carbohydrates by different tissues or cells.

The results obtained in the treatment of the induced mammary carcinoma suggest that the arguments presented for the performance of this work are plausible. However, it is difficult to explain why only 14 treated rats did not develop tumors and why the tumors did not disappear from the rest.

Among other things, it is possible that in some rats (although they are homozygous), the inhibitor did not reach the site of the tumor owing to enzymic inactivation or nonspecific reaction with other compounds containing -SH groups. However, even in these rats it is possible to distinguish histopathologically the effect of the inhibitor by examination of the histological pattern of the carcinomas from treated and untreated rats (45). In any case 33% of the treated rats were free from tumors, and in the 67% the number and size of the tumors were significantly less than in controls.

RESUMEN

Se ha postulado que el tejido tumoral muestra una actividad glicolítica elevada debido a la baja actividad de la glicerol fosfato deshidrogenasa y de la β -hidroxibutirato deshidrogenasa; al mismo tiempo se encuentra una elevación de la deshidrogenasa láctica (DL). Por lo tanto, la inhibición específica de la DL puede abrir una nueva posibilidad a la inhibición del crecimiento tumoral. Se diseñó y sintetizó, de acuerdo con las ideas de Baker de los "antimetabolitos no clásicos" el compuesto *N*-(4-carboxi-2-hidroxi-fenil) malcímida. Este preparado actúa como inhibidor irreversible de la DL mostrando una K_i de 1×10^{-4} moles \times $1t^{-1}$. El oxamato (un inhibidor competitivo de la DL) protegía a la enzima contra la inactivación irreversible lo que

parece ser una inhibición del sitio activo. Cuando se dializaba a la DL inhibida por maleimida, no se obtenía la recuperación de la actividad enzimática, pero cuando la DL inhibida con oxamato era dializada, se obtenía una reactivación total.

El tejido tumoral muestra una elevación de la isozima M de la DL y un inhibidor específico de esta isozima podría poseer actividad antitumoral. La maleimida es este inhibidor. El ensayo biológico mostró los siguientes resultados: no se encontró actividad contra el tumor de ascitis y el sarcoma 180. Por otra parte, en ratas Sprague-Dawley portadoras de carcinoma mamario provocado con 7,12-dimetilbenzantraceno, la maleimida inhibió significativamente la aparición y el desarrollo del tumor.

Esta inhibición de la DL puede usarse con ventaja en estudios sobre la participación de esta enzima en la regulación del metabolismo de los carbohidratos.

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DISCUSSION ¹

Discussor, DR. H. A. LARDY, The University of Wisconsin, Institute for Enzyme Research, Madison, Wisconsin

THE six papers presented in this session covered so wide a variety of subjects that no unifying thread was apparent, and the discussion therefore dealt with the papers individually. Dr. Lardy pointed out that Dr. Fischer's disclosure of a regulatory role for glucose-6-phosphate in controlling phosphorylase activity furnishes evidence that all of the pathways providing carbohydrate to a cell may be regulated by this common sugar phosphate ester. Hexokinase and partially phosphorylated phosphorylase are inhibited by glucose-6-phosphate, whereas one form of glycosyl transferase is stimulated by glucose-6-phosphate. Of crucial importance is the fact that the regulation of phosphofructokinase activity can determine the steady-state concentration of glucose-6-phosphate. Rapid phosphorylation of fructose-6-phosphate results in diminished concentrations of glucose-6-phosphate and consequently enhances the rate of formation of hexose monophosphate from either free glucose or glycogen.

Dr. Osvaldo Cori inquired about possible mechanisms for regulating phosphoglucomutase but Dr. Edmund Fischer indicated that none was known other than the possibility of converting the enzyme from the dephospho- to the phosphorylated form. Dr. del Rio discussed how glucose-1,6-diphosphate concentration may regulate phosphoglucomutase activity. Prof. Handler's group has found a variety of phosphoglucomutases in different living forms; some exhibit a sequential mechanism, others a "Ping-Pong" mechanism. In answer to an inquiry from Dr. (Miss) Gonzalez, Dr. Fischer indicated that some preparations of rabbit liver phosphorylase show differences in activity in the presence and absence respectively of glucose-6-phosphate, but the differences are not nearly as great as are observed with the muscle enzyme.

¹ Of articles by Edmond H. Fischer, Suzanne S. Hurd, Pearl Koh, and David Teller; J. Moura Gonçalves and A. Focesi, Jr.; and Raúl N. Ondarza; G. Cilento and K. Zinner; A. Gómez-Puyou, M. Tuena, and A. Peña; and Guillermo Carvajal, Enedina J. Carvajal, Ricardo Yáñez, and Virginia Z. Medina.

The discussion brought out that Dr. Gonçalves' interesting observation of a competitive relationship between dinitrophenol and adenylic acid in their effects on muscle phosphorylase does not necessarily indicate that these two compounds are binding at the same site. Dr. Fischer had tested ribose-5-phosphate as a compound that resembled both glucose-6-phosphate and AMP, but found it completely without effect on phosphorylase *b*.

Dr. Lardy offered an hypothesis to explain Dr. Ondarza's observed inhibition of glutathione reductase by the mixed disulfide, CoASSG. The enzyme is presumed not to react with oxidized glutathione or the mixed disulfide unless the dithiol group on the enzyme has been reduced by pyridine nucleotide. When the reduced enzyme reacts with CoASSG, GSH is liberated and the mixed disulfide of enzymes-SCoA is formed. The bulky CoA residue may prevent access of the enzyme thiol to the mixed disulfide and thus prevent release of CoASH and formation of oxidized enzyme. On the other hand, when reduced pyridine nucleotide is added last, the oxidized form of the enzyme is reduced in the presence of a mixture of substrate (GSSG) and inhibiting substrate (CoASSG) where GSSG may compete effectively for the substrate site and be reduced with little evidence of inhibition. A test of this hypothesis would be to treat oxidized enzyme with reduced coenzyme A and Dr. Ondarza indicated this had been tried but no inhibition was obtained.

Comparisons were made of Dr. Cilento's observations of catechol enhancement of *p*-hydroquinone oxidation with Dawson's findings that enzymic oxidation of monophenols is enhanced by diphenols.

Dr. Gómez-Puyou and his questioners discussed the significance of potassium transport into mitochondria against a gradient in view of the fact that under physiological conditions the mitochondria are already in a high K^+ environment.

TUESDAY MORNING

Chairman: Manuel V. Ortega

Cooperative Phenomena and Conformational Changes^{1,2}

D. E. KOSHLAND, JR., and M. E. KIRTLEY, *Biochemistry Department, University of California, Berkeley, California, 94720*

SUMMARY

The idea that the active sites of many enzymes must be flexible for their proper functioning initially arose from kinetic data. Evidence from reactivity of amino-acid side chains, difference spectra, optical rotatory dispersion, and X-ray crystallography has been obtained to support this concept. Since the direct evidence of protein structure is consonant with the ideas of conformational changes during enzyme action, we have recently reexamined the kinetics of these systems, particularly because of their importance in metabolic control. Many enzymes involved in control are composed of subunits, and subunit interactions, therefore, are an added factor to be considered in the conformational changes induced by small molecules. A model capable of explaining a wide variety of phenomena has been developed by considerations of the interactions of various ligands with a subunit and then extension of these considerations to proteins

involved with subunits. The basic features of the model are that the ligand induces in the subunit a conformational change which may or may not perturb the adjacent subunits. If the interaction between the subunits is taken into account, an explanation for cooperative effects leading to kinetics which deviate from Michaelis-Menten kinetics is obtained. By the use of computer programs, it is possible to consider the effects of these interactions on the over-all kinetics and to develop simple diagnostic tests to delineate the structural changes occurring during the action of feedback enzymes. By observation of the qualitative effects of parameters, such as the maximum velocity, the midpoint of the saturation curve, and the steepness of activity curve, it is possible to draw conclusions about conformational changes induced by ligands, compulsory orders of binding, and the extent of subunit interactions.—*Nat Cancer Inst Monogr* 27: 129-140, 1967.

IT IS NOW widely accepted that enzymes cannot be considered in the classical template sense, but rather must be viewed as flexible molecules whose conformational states are vital to the understanding of

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. J. P. Changeux, p. 165.

enzyme activity. The initial evidence for such a concept came from kinetic studies (1-3), but in the ensuing years chemical and physical tools—such as the reactivity of amino acid residues, difference spectra, fluorescence spectra, nuclear magnetic resonance, and X-ray crystallography—have provided strong evidence to demonstrate that the conformational state of the protein is influenced by the small ligands bound to it. To pick a few recent examples, the elegant studies of O'Sullivan and Cohn have combined kinetics, amino acid analyses, and nuclear magnetic resonance to correlate conformational changes with the specificity pattern of the protein (4). Spin resonance has also enabled Berliner and McConnell (5) to provide evidence for conformational change and to place some kinetic limits on the lifetimes of the various conformational states. In addition X-ray crystallography by Perutz, Phillips, and their coworkers has provided precise measurements on the movement of amino acid side chains of hemoglobin and lysozyme under the influence of small molecules (6, 7).

These conformational properties, moreover, are not academic curiosities. They apparently are of key importance in the biological effectiveness of enzymes in phenomena varying from specificity control, through the sequential binding of substrate, the temperature coefficients of enzymes, the aggregation of proteins, to the widely discussed feedback control enzymes. The detailed study of feedback control, pioneered by Umbarger (8), Gerhart and Pardee (9), and Monod, Changeux, and Jacob (10) has been one of the most exciting and significant developments in modern biochemistry.

Because of the large amount of work in this area, we shall not attempt a review of the field. Excellent reviews are numerous, and two recent ones which are comprehensive and perceptive are those of Atkinson (11) and Stadtman (12). We shall, therefore, turn our attention to some aspects in the correlation of the structural changes in the protein with its kinetic properties. More specifically, we shall be concerned with the relation of the subunit structure of the proteins to the cooperative binding phenomena observed in so many feedback enzymes.

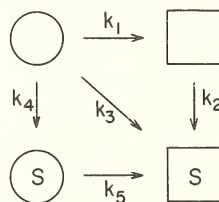
Two models of apparently wide applicability have arisen to explain these properties. One of these is the model of Monod, Wyman, and Changeux (13) which postulates: (a) that an equilibrium exists between different conformational states of the protein, (b) that the conformations of all protein subunits change simultaneously, and (c) that there is a differential affinity of small ligands for one state of the protein relative to the other. Another model to explain the cooperative effects has been proposed in our laboratory [Koshland, Némethy, and Filmer (14)] and it involves three basic postulates: (a) The substrate induces a conformational change which may be limited to a single subunit of the protein, (b) the conformational changes in the indi-

vidual subunits occur sequentially rather than simultaneously, and (c) a differential binding affinity for ligands exists between one conformational state and the other of the protein.

It has already been shown that either of these models is capable of explaining the affinity curves of oxygen to hemoglobin quantitatively and that many of the predictions resulting from these structural models are compatible with studies on enzyme action (14-16). It becomes increasingly important therefore to examine the physical forces that would lead to one model or the other and also to develop diagnostic tests which will distinguish between potential mechanisms in a given experimental situation.

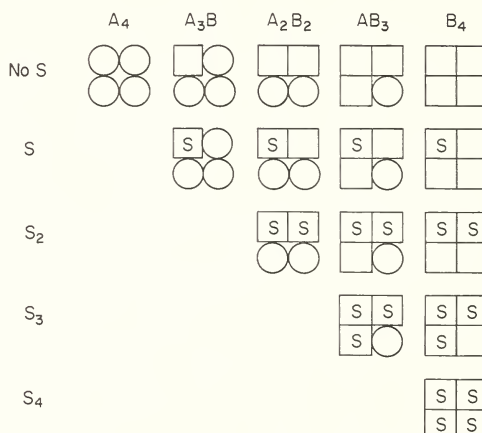
Before discussing the subunit interactions, we might consider the mechanism by which a conformational change in a given subunit might be achieved under the influence of a small ligand. Text-figure 1 shows three alternate pathways. In one extreme a conformational change precedes the binding of substrate (k_1), and this is followed by binding of substrate to the new conformation (k_2). Another extreme is the initial binding of substrate to the new conformation change (k_4) followed by a conformational change (k_5). An intermediate between these extremes would be a pathway in which the binding of substrate and the conformational changes occur continuously. The thermodynamics of the final state will be the same regardless of the pathway, *i.e.*, $K_s K_t = K'_s K'_t$, where K_t , K_s , K'_s , and K'_t are the equilibrium constants for steps k_1 , k_2 , k_4 , k_5 , respectively. For simplicity we shall use K_s and K_t to describe the thermodynamic states of the over-all process without implying that this represents a preferred kinetic pathway.


TEXT-FIGURE 1.—Pathways for the formation of a new conformational state under the influence of a ligand S.



Text-figure 2 shows a generalized model which may be helpful in illustrating the forces involved in subunit interactions. We shall use the notation described previously (14). In this discussion we will use a tetrameric protein as an example, but the model is equally applicable to proteins of any number of subunits. The subunit conformation usually existing in the absence of any bound ligand is designated by A or by a circle. The subunit conformation induced or stabilized by a bound ligand is designated by B or by a square. K_{AA} , K_{AB} , and

K_{BB} represent the equilibrium constants which measure the relative strength of interaction between adjacent subunits in the AA , AB , and BB conformations, respectively.



TEXT-FIGURE 2.—Generalized model to illustrate factors involved in more simplified models. Conformations A and B are designated as *circle* and *square*, respectively. Combinations are illustrated for a tetrameric protein, in which S is bound in significant quantities only to the B conformation. The form  is omitted from the A_2B_2 species for simplification of the presentation, but is included in the calculations.

If one defines AA as the standard state ($K_{AA} = 1$), values of K_{AB} and K_{BB} greater than 1 reflect the fact that the strengths of the AB and BB interactions are greater than those of the interactions between subunits in the AA conformation. Values of K_{AB} and K_{BB} less than 1 indicate that these interactions are less than for the AA interaction. K_{AA} can ultimately be related to dissociation constants for the protein into subunits, but in these studies only the relative changes in subunit affinities are of interest, and hence the definition of a standard state involves no loss in generality.

Table 1 shows the relative amounts of various species for certain selected values of K_S , K_T , K_{AB} , and K_{BB} . In the absence of substrate the protein exists essentially in the A_4 form in examples 1, 2, and 3 in the table. Since no substrate is present and the transformation from A to B is not favored energetically, the favorable BB interaction (10 times as great as AA) is not sufficient to overcome the unfavorable energy of the conformation change ($K_T = 10^{-2}$), and therefore the protein is largely in the A_4 form in the absence of ligand. If, however, the BB interaction (adjacent squares in the text-fig.) is very favorable, *i.e.*, 1,000 times more attractive than the AA interaction, even

TABLE 1.—Distribution of molecular species on binding of ligand to a tetrameric protein with conformational changes
Assumption: $K_S = 10^3$, $K_{AA} = K_{AB} = 1$, S bound only to B conformation

Example	K_T	K_{BB}	Distribution of conformational states of proteins in absence of S					Distribution of conformational states of proteins at $Y = 0.5^*$					R_S^\dagger
			of proteins in absence of S										
			A_4	A_3B	A_2B_2	AB_3	B_4	A_4	A_3B	A_2B_2	AB_3	B_4	
1	10^{-2}	10	96	4	0	0	0	25	11	13	13	27	7
2	10^{-3}	10	99.6	.4	0	0	0	30	12	13	12	31	6
3	10^{-4}	10	100	0	0	0	0	31	12	13	12	31	5
4	10^{-2}	10^3	0	0	0	.04	99.9	0	0	0	0	100	81
5	10^{-3}	10^3	50	.2	.2	.2	50	6	0	0	0	94	49
6	10^{-4}	10^3	100	0	0	0	0	40	0	0	0	60	4

*Conformational state of the protein includes the sum of all forms containing the indicated molecular species. Thus A_2B_2 is the sum of A_2B_2 , A_2B_2S , and $A_2B_2S_2$. The total moles of S bound to any species can never exceed the total subunits in the B conformation when exclusive binding to B only is assumed. Y is the fractional saturation of total sites.
 $^\dagger R_S$ is the ratio of the ligand concentration at 90% saturation to that at 10% saturation. A value of 81 is characteristic of a Michaelis-Menten curve.

in the absence of substrate, amounts of the B_4 form are sizable. If the energy is not too unfavorable ($K_T = 10^{-2}$), all protein will shift to the B_4 form (example 4, table 1). If it is quite unfavorable, only part of the protein will shift (example 5). Thus, when $K_T = 10^{-3}$ and $K_{BB} = 10^3$, one half of the protein is found as B_4 and one half as A_4 in the absence of substrate.

When the substrate is added, the B conformation will be favored further and a shift occurs from the A to the B conformation in many or all of the subunits. At the midpoint of the saturation curve, *i.e.*, when Y , the fractional saturation, is 0.5, we have calculated the actual species which would be present in solution. In examples 1, 2, and 3, molecular species containing all possible mixtures of conformations are present, *i.e.*, A , A_3B , A_2B_2 , AB_3 , and B_4 . In examples 5 and 6, only the species A_4 and B_4 are present in any significant amounts. The former examples therefore correspond to the model of Koshland, Némethy, and Filmer where hybrid species are allowed. The latter correspond to the model of Monod, Wyman, and Changeux in which all subunits are observed to change in a concerted manner. The calculations reveal what might have been guessed intuitively, *e.g.*, other factors being equal, very strong subunit interactions will lead to the concerted model of Monod, Wyman, and Changeux, and relatively weak subunit interactions will lead to the simplest hybrid species model of Koshland, Némethy, and Filmer.

The parameter R_s measures the cooperativity of the interactions. It has been defined as the ratio of substrate concentration at 90% saturation to that at 10% saturation. This ratio is 81 for all Michaelis-Menten curves regardless of K_s . It is observed, of course, in all cases in which there are no subunit interactions or in which the protein remains in one conformation during enzyme action, *e.g.*, example 6 in table 1. For curves which are steeper or more cooperative than the Michaelis-Menten curve, these values will be <81 ; the smaller the value the greater the degree of cooperativity. Examples 3 and 6 are of approximately equal cooperativity, but exhibit quite different distribution of molecular species. Thus, the two models can give very similar over-all saturation curves, but could be distinguished from each other if direct measurements of the conformations of subunits could be obtained. The reporter group technique is a possible tool for much direct measurement of conformations (16).

A priori it is not possible to state how strong the relative subunit interactions are likely to be in a given protein. If the subunit interactions always lead to molecular species of the A_4 and B_4 type only, then the Monod, Wyman, and Changeux model will be the predominant one in feedback enzymes. If the interactions lead to intermediate forms of types A_3B , A_2B_2 , etc., then the Koshland, Némethy, and Filmer model involving hybrids will apply. If the situation varies from pro-

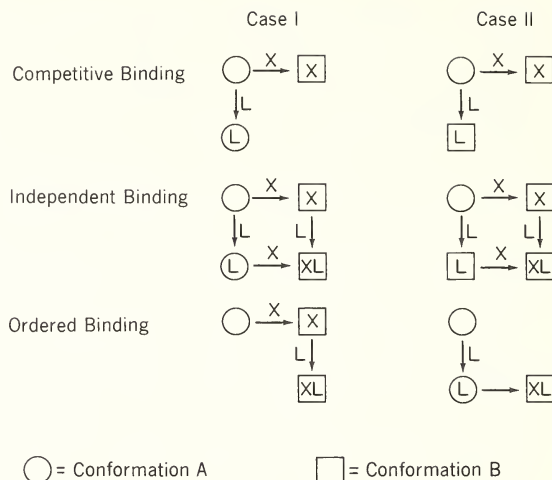
tein to protein, then experimental tools will be needed to determine which mechanisms apply in each of the individual cases.

The latter situation seemed most likely to us, and therefore it was important to develop diagnostic tests to evaluate alternative mechanisms. Equilibrium dialysis, X-ray crystallography, fluorescence probes, etc., are possible tools. Each has its values, but development of kinetic criteria seemed particularly desirable, since so much kinetic data are already available. We asked three questions in beginning such an inquiry. Can diagnostic tests be developed involving the parameters normally measured in enzyme studies? Can relatively high discrimination between mechanisms be obtained without elaborate measurements? Do mechanisms lead to predictions which can be tested? Fortunately, the answer to all three questions has been a qualified "Yes." The detailed derivations and applications will be reported elsewhere (15), but an outline of the procedure and results is given here.

In the first place, all the reasonable interactions at a subunit were considered, assuming two conformational states of each subunit as far as intersubunit interactions were concerned. These possibilities are shown in text-figure 3, where for simplification only the effects on a single subunit are shown. For example, in the competitive I mechanism, one of the ligands, X , is illustrated as binding with a consequent change of subunit to the B conformation, whereas L binds to the A conformation. In case II, both X and L lead to a conformational change in the subunit. After the mathematical equations are derived for these two cases, X and L can be designated as substrate and inhibitor, substrate and activator, or substrate and second substrate. Moreover, with any two of these ligands, some of the mechanisms will lead to additional alternatives. Thus, in the competitive I case, the implications are different if X is a substrate and L is an inhibitor and when L is the substrate and X is the inhibitor, because X has been designated as the ligand which induces the conformational change. Calculations for both types of mechanisms were therefore made in this case. In the competitive II case it is clear that the alternative designations lead to precisely the same mechanism, since both X and L induce conformational changes. A brief consideration along these lines will show that all of the possible permutations are included in table 2 for these three reasonable types of interactions.

The ligand saturation curves were then calculated in the manner described previously (13) for a tetrameric protein for each of the possible mechanisms shown in text-figure 3. With the aid of the computer, the effect on V_{max} (V_M), $S_{0.5}$, and R_S of increasing the concentration of the second ligand was calculated. The results with an activator and a substrate are shown in table 2. There it is seen that the observations—that V_M is unchanged, $S_{0.5}$ decreases, and the cooperativity goes from Michaelis-Menten (81) to cooperative (<81) on increasing acti-

TEXT-FIGURE 3.—Possible mechanisms at individual subunits as a result of the binding of two ligands, X and L . X and/or L may represent substrate, activator, or inhibitor. The individual subunit may be part of a polymeric enzyme.



vator concentrations (line 5 in table 2)—are compatible with an Ordered II mechanism only. On the other hand, the same effect on V_{M} and $S_{0.5}$ with an R_s change from <81 to 81 (line 2 in table 2) would be compatible with the Independent II, Ordered I, and Monod, Wyman, and Changeux models. Thus, this set of tests in all cases reduces the number of alternatives which must be considered, but does not produce a “unique” mechanism in all cases. A brief glance at the table shows that two parameters would, of course, provide considerably less discrimination; four parameters would, of course, provide more, but then greater work would be required to obtain the necessary data. The three parameters chosen at least as an initial probe seemed a reasonable compromise between degree of discrimination and effort involved.

Similar tables have been compiled for the interactions of substrates and inhibitors and also for two substrates. If the results with one set of ligands, *e.g.*, substrate and activator, lead to several alternatives as discussed above, a second set of experiments with two other ligands, *e.g.*, substrate and inhibitor, may help to choose between these alternatives. Moreover, tests for internal consistency can be made from such comparisons. For example, the substrate-plus-inhibitor tests may yield a unique solution in which substrate induces a conformational change to the B conformation. Therefore, at least one of the alternatives in the substrate-plus-activator experiments must include such a mechanism. It has been found in this way that in many cases a single consistent over-all mechanism for a given enzyme can be deduced from the analysis.

The fact that we were able to obtain a high level of discrimination by such a few tests is encouraging. It should be emphasized, however, that this is by no means sufficient to establish a given mechanism. To

TABLE 2.—Possible mechanisms compatible with observed changes in V_M , $S_{0.5}$, and R_S on increasing concentration of an activator molecule

Change in observed parameters for the substrate in absence and presence of activator (<i>A</i>)				Model	Equation	Activator	K_{BB}^*
V_{max}	$S_{0.5}$	R_S					
		No <i>A</i>	With <i>A</i>				
No change	Decrease	81	81	Ind II Ord I Ord II	N_{X_t} N_{X_t} (or N_{L_t}) N_{X_t} (or N_{L_t})	L L (or X) L (or X)	1 1 1
No change	Decrease	<81	81	Ind II Ord I Monod <i>et al.</i> †	N_{X_t} N_{L_t}	L X	>1 >1
No change	Increase	<81	81	Comp II‡ Monod <i>et al.</i> †	N_{X_t}	L	>1
No change	Decrease	<81	<81	Ord I Ord II	N_{X_t} N_{X_t}	L L	>1 >1
No change	Decrease	81	<81	Ord II	N_{L_t}	X	>1
Increase	No change	81	81	Ind I Ind I	N_{XL} (or N_{LX}) N_{LX}	L (or X) X	1 >1
Increase	No change	<81	<81	Ind I	N_{XL}	L	>1
Increase	Decrease	81	81	Ind II Ord I Ord II	N_{XL} N_{XL} N_{XL}	L L X	1 1 1
Increase	Decrease	<81	81	Ind II	N_{XL}	L	>1
Increase	Decrease	<81	<81	Ord I Ord II	N_{XL} N_{LX}	L X	>1 >1

*The value of K_{BB} when K_{AB} equals 1.† Also included are the effects predicted by the model of Monod *et al.* (13) assuming exclusive binding.

‡ Activation only at low concentrations of "activator" and substrate; inhibition at higher concentrations of either ligand.

obtain the proper kinetic equations, certain simplifying assumptions had to be used, such as the assumption of rapid reversible equilibria prior to the rate determining liberation of product. The indicated mechanisms, therefore, strictly apply to only those enzyme systems which fulfill these assumptions. The assumptions chosen are reasonable and probably pertinent for many enzymes, but because of them, the deduced mechanism must be considered as a working hypothesis to be confirmed by further studies. The advantage of the mechanisms deduced here, however, is that they can explain a great deal of the accumulated data and they do lead to quantitative predictions in regard to other kinetic experiments and to subunit conformations such as

those in table 1. These predictions can be tested. Therefore, such a diagnostic test system is one step toward the establishment of a definitive mechanism for a particular enzyme. It is encouraging that this step can be achieved without an unreasonable expenditure of time and with the use of parameters which are already known to be measurable.

Clearly, certain assumptions in specific situations cannot be generalized, and it is important not to confuse the detailed observations of an individual case with the essential assumptions of a model. For example, it is an essential feature of both models that there is a preferential affinity to one of the conformations. It is not essential that this preferential affinity result in an exclusive binding to one conformation, although this extreme may occur in certain cases. Thus, exclusive binding, *e.g.*, the observation that no appreciable concentrations of ligands are found bound to conformation A, has been found to be a satisfactory assumption in the application of the Koshland, Némethy, and Filmer model to hemoglobin and the Monod, Wyman, and Changeux model to aspartyl transcarbamylase. It would, of course, simplify calculations if such an exclusive binding were a universal phenomenon, but *a priori* there is no reason to justify such a general assumption. The consequences of nonexclusive binding in certain cases have been considered by Rubin and Changeux (17), and similar considerations can be applied, when justifiable, to other models.

Models such as those outlined here are being developed to relate the ligand binding and kinetic properties of proteins to the structure and conformation of the protein subunits. Not only do such models provide a simple basis for correlating a wide variety of data, but, perhaps more importantly, they present a basis for predicting other properties of the proteins which can be tested experimentally. The determination of the molecular details of the processes by which these control phenomena of proteins are achieved would soon represent another advance in our understanding of the way in which control is achieved in biological systems.

RESUMEN

La idea de que el sitio activo de muchas enzimas deba ser flexible para que funcione adecuadamente se originó, en un principio, en datos cinéticos. En apoyo de este concepto se han acumulado pruebas basadas en datos de reactividad de las cadenas laterales de los aminoácidos, de espectros diferenciales, de dispersiones ópticas rotatorias y cristalografía con rayos X. Como las pruebas directas de la estructura proteica están de acuerdo con las ideas de los cambios conformacionales durante la acción enzimática, recientemente hemos vuelto a examinar la cinética de estos sistemas, sobre todo en vista de su importancia en el control del metabolismo. Se ha observado que muchas de las enzimas que intervienen en el control metabólico están compuestas por subunidades y, por lo tanto, las interracciones de subunidades

constituyen un factor sobreañadido que debe tenerse en cuenta con respecto a los cambios conformacionales provocados por moléculas pequeñas. Se ha desarrollado un modelo capaz de explicar una gran diversidad de fenómenos, considerando las interacciones de varios ligandos con una subunidad y después extendiendo estas consideraciones a proteínas relacionadas con subunidades. Los hechos sobresalientes de este modelo son que el ligando provoca un cambio conformacional en la subunidad y que este cambio conformacional puede o no perturbar las subunidades adyacentes. Si se toman en cuenta las interacciones entre las subunidades, se obtiene una explicación de los efectos cooperativos que llevan a un tipo de cinético que se desvía de la de Michaelis-Menten. Por medio de programas de computadora es posible considerar el efecto de estas interacciones sobre la cinética global y desarrollar pruebas diagnósticas sencillas para delinear los cambios estructurales que ocurren durante la acción de enzimas de retroalimentación. El estudio de los efectos cualitativos de parámetros tales como la velocidad máxima, el punto medio de la curva de saturación y la pendiente de la curva de actividad, permite derivar conclusiones sobre los cambios conformacionales provocados por los ligandos, los órdenes obligados de unión y el grado de las interacciones de las subunidades.

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Solvent Effects on Reactions Catalyzed by Some Proteolytic Enzymes^{1,2}

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SUMMARY

It has been shown that the dielectric constant (D) and the ionic strength (μ) of the solvent influence the velocity of reactions catalyzed by trypsin, α -chymotrypsin, and other proteinases. Above pH 7, the effect of both D and μ on esterolysis catalyzed by either trypsin or α -chymotrypsin is distinct. The effect of μ on esterolysis catalyzed by the plant proteinase mexicain is similar to that exerted on systems involving α -chymotrypsin. In trypsin-catalyzed esterolysis of substrates containing a free α -ammonium group, at pH values below the pK_a of the substrate, a transesterification reaction between the substrate and alcohol overrides the dielectric effect of the latter resulting in an apparent inhibition. However, the

actual influence of D can be ascertained by use of an aprotic solvent like acetone. Trypsin- and α -chymotrypsin-catalyzed hydrolysis of casein behave like their respective counterparts in esterolysis as far as the effect of μ is concerned. The effect of μ on proteolysis of urea-denatured hemoglobin differs from that on casein hydrolysis. Apparently neutral salts increase the denaturing effect of urea on both hemoglobin and the proteinases. Since trypsin and α -chymotrypsin are very sensitive to urea, their proteolytic activity decreases as μ increases. The proteolytic activity of mexicain increases in the presence of urea and with an increase of μ .—*Nat Cancer Inst Monogr* 27: 141–152, 1967.

THE RATE of chemical reactions involving ions or dipolar molecules is modified by a change in the dielectric constant or the ionic strength of the medium. Several approaches have been developed to account for solvent effects on reaction rates insofar as the medium dielectric constant is concerned, *e.g.*, those of Scatchard (1), Laidler and Eyring (2), and Amis (3). The basic treatment of salt effects on ionic re-

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. J. P. Changeux, p. 165.

actions is mainly due to Brønsted (4), even though it has been further revised by several authors, for instance La Mer (5, 6), or Amis and Jaffé (7) in the subject of ion-dipole reactions. Accordingly, since enzyme molecules possess ionizable groups, some of which play a role in their catalytic function, it is to be expected that the rates of enzymic reactions vary with both the dielectric constant and the ionic strength of the medium. As a matter of fact this has been repeatedly observed, but the interpretation is subject to controversy. Most commonly a variety of specific interactions is invoked to account for solvent effects on enzyme-catalyzed reactions. Some examples follow: Inagami and Sturtevant (8), in an attempt to explain the activation and subsequent inhibition of the trypsin-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) in dioxane-water media, arrived at the conclusion that the effects are due to changes in the concentration of water. Bender *et al.* (9) consider that the effect of nucleophiles such as methanol, ethanol, and hydroxylamine, on α -chymotrypsin-catalyzed reactions can be interpreted in terms of a competition of water and the nucleophile for the acyl-enzyme intermediate compound. For the effect of aprotic solvents like dioxane, acetone, and acetonitrile on the kinetics of α -chymotrypsin-catalyzed hydrolyses, Clement and Bender (10) utilized a treatment involving the combination of the dielectric effect and simple competitive inhibition. However, more recently, Bender and Kézdy (11) proposed a mechanism of action of α -chymotrypsin, applicable as well to trypsin, in which according to the authors "... all the transition states should be neutral, predicting no effect of ionic strength or dielectric constant on the rates, as found experimentally."

On the other hand, Laidler (12) predicted that an enzymic reaction will be accelerated or slowed down by an increase in dielectric constant, depending upon whether the activated complex is more or less polar than the reactants. He studied two examples: the α -chymotrypsin-catalyzed hydrolysis of methyl hydrocinnamate (13) and the myosin-catalyzed hydrolysis of ATP (14), both of which behaved in agreement with the theoretical expectations. Castañeda-Agulló and del Castillo (15-19), under conditions which allowed to eschew specific effects, showed that there is a common effect of a variety of organic additives on the rate of hydrolysis of esters catalyzed by either trypsin or α -chymotrypsin, and that this effect can be related to the change in the medium dielectric strength. The effect of a variation of dielectric constant on these reactions is of opposite trend depending on the enzyme involved, which, according to theory (3), indicates the intervention of distinct charges in every case. In the same way, Castañeda-Agulló *et al.* (20, 21) could observe that the kinetic effects, resulting from changes in the ionic strength, on trypsin- and α -chymotrypsin-catalyzed hydrolyses of esters are amenable to the same electrostatic

treatment applicable to chemical reactions. The conclusion was then reached that salt effects are mainly due to changes in the dissociation of ionizable groups which are not the same in the case of trypsin as in that of α -chymotrypsin.

Whitaker, Tappel, and Wormser (22) also found a correlation between the rate of α -amylase-catalyzed hydrolysis of starch in the presence of various organic additives and the medium dielectric constant. The same authors (23) observed that there is a relationship between the rate of reactions catalyzed by α -amylase, alcohol dehydrogenase, and hematin and the ionic strength, similar to those observed in reactions catalyzed by trypsin and α -chymotrypsin.

It has been reported by Smith *et al.* (24) that the hydrolysis of benzoyl-L-argininamide by papain is inhibited by alcohols and that this inhibition may be explained by effects on the dielectric constant of the medium.

Ono, Hiromi, and Sano (25), in an investigation of the effect of methanol on the kinetics of crystalline bacterial α -amylase, after examining various possible effects, reached the conclusion that the dielectric constant effect was predominantly operative. Likewise, Hiromi (26), who also holds the opinion that enzymic reactions must be influenced by the dielectric constant of the medium in the same ways as are reactions involving small ions or molecules, has developed a treatment for the effect of the dielectric strength on enzyme kinetics. This is based on a model of Kirkwood (27) for the charge distribution in the enzyme molecule.

We shall discuss here the evidence in favor of the influence on enzymic reactions of the dielectric constant and the ionic strength through modified electrostatic interactions between enzyme and substrate.

It has been shown that in the trypsin-catalyzed hydrolysis of BAEE (15) or *p*-toluenesulfonyl-L-arginine methyl ester (TAME) (28), and within a given range of dielectric constant (D), the plot of the logarithm of the rate constant *versus* the reciprocal of D is a straight line of positive slope. The linearity is in accordance with the expressions given below which were derived by Amis (3) from the Coulomb's law and Arrhenius' equation to account for the influence of the dielectric constant in three types of chemical reactions: ion-ion [1], ion-dipolar molecule [2], and dipolar molecule-dipolar molecule [3]. In all three cases the equations predict a linear relationship between $\log k'$ and $1/D$, *viz.*

$$k'_D = k'_\infty e^{-(z_1 z_2 e^2 / D k T r)} \quad [1]$$

$$k'_D = k'_\infty e^{-(z e \mu / D k T r^2)} \quad [2]$$

$$k'_D = k'_\infty e^{-(2 \mu_1 \mu_2 / D k T r^3)} \quad [3]$$

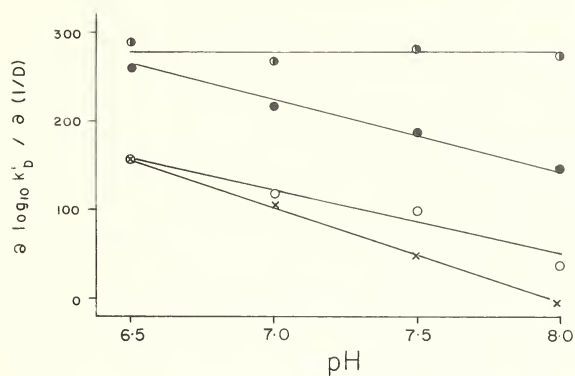
In these, k'_D and k'_∞ stand for rate constants at a given D value and at infinite dielectric constant where electrostatic interactions vanish, z stands for ion valence, e for electronic charge, μ for dipole moment, k for Boltzmann constant, T for absolute temperature, and r for reacting distance.

The sign of the slope in the system trypsin-BAEE or trypsin-TAME is consistent with either an interaction between oppositely charged ions or one between a cation and a dipole. The trend of the dielectric effect on the above systems remains constant from pH 5.5–8.5 (17); the reaction is accelerated in media of low dielectric constant and vice versa. Not all the solvents which lower the dielectric constant are equally effective; *e.g.*, ethanol and methanol function better than dioxane (29). In the α -chymotrypsin-catalyzed hydrolysis of esters at pH 7.8 (16), the trend of the dielectric effect is the opposite to that observed with trypsin at the same pH (15): the rate increases as D increases, by addition of, *e.g.*, urea (17) or glycine (19). However, as pH diminishes the effect is reverted in the vicinity of 7, so that in the acid region the behavior of both enzymes is very similar.

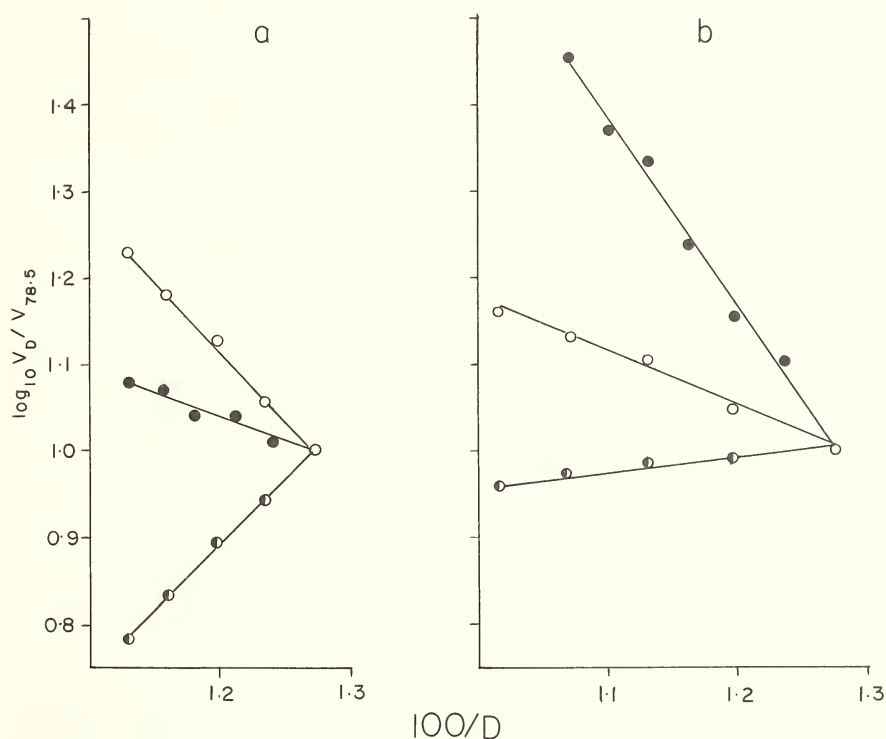
The effect of various additives on the trypsin-catalyzed hydrolysis of TAME is summarized in text-figure 1. From pH 6.5–8, the effect of *n*-propanol is constant within experimental variations and the highest of the group. Those of acetone and urea tend to decrease as pH increases, however, remaining positive. The effect of glycine at pH 6.5 is equal to that of urea, but diminishes more rapidly and becomes practically nil at pH 8. While there is no obvious reason for the abatement of the effect of either acetone or urea with pH increment, the reduction in the effect of glycine can be explained on the basis of a previous finding of Dunning and Shutt (30). They observed that the increase in D , when glycine is dissolved in water, depends on the relative proportion of dipolar ions, thus being maximum from pH 4.5 to 7.5 and falling steeply outside this range.

The effect of urea or glycine on the α -chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester (PEE) changes from inhibitory to enhancing as pH increases with a transition between 6.5 and 7 (17, 19). Text-figure 2 shows that the extent of the effect of urea, both inhibitory and enhancing, is greater than that of glycine at pH 6.25 and 7.5. However, at pH 8, glycine exerts the greater enhancing effect. As already suggested (19), glycine may modify the reaction rate not only by increasing D , but also because of its dipolar ion structure it might strengthen centers of charge. Again, the possibility cannot be excluded that glycine and/or urea bring about structural effects favorable or unfavorable to the enzymic action that contributes to the quantitative difference in their effects.

The effect of ionic strength on the esterolytic activity of three proteinases is shown in text-figure 3. Mexicain is a crystalline plant pro-

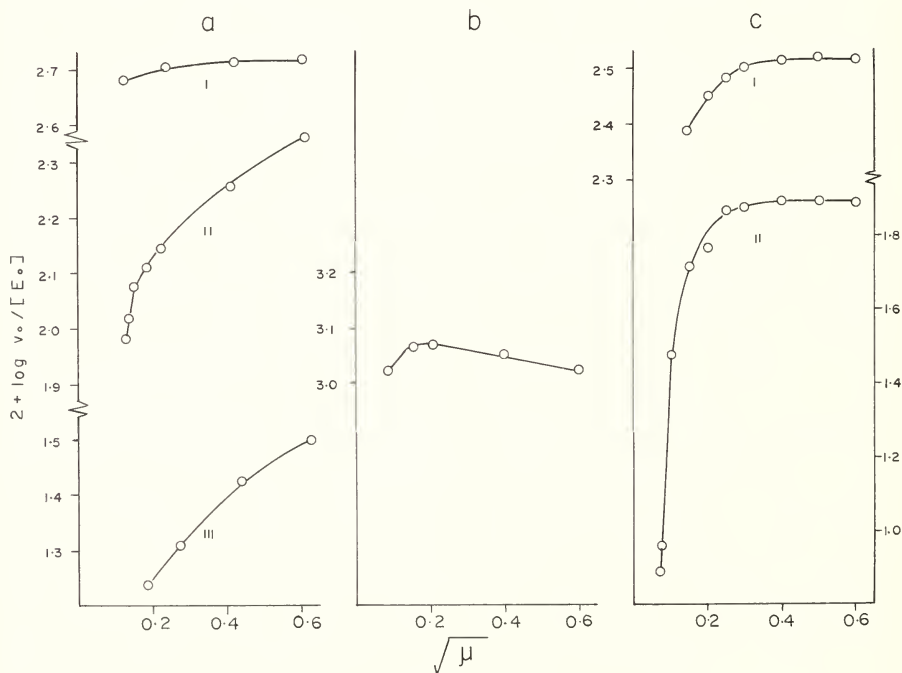


TEXT-FIGURE 1.—Change in the rate constant logarithm with the reciprocal of dielectric constant as a function of pH for the system trypsin-TAME at 25 C in aqueous: n -propanol (●), acetone (●), urea (○), and glycine (x).



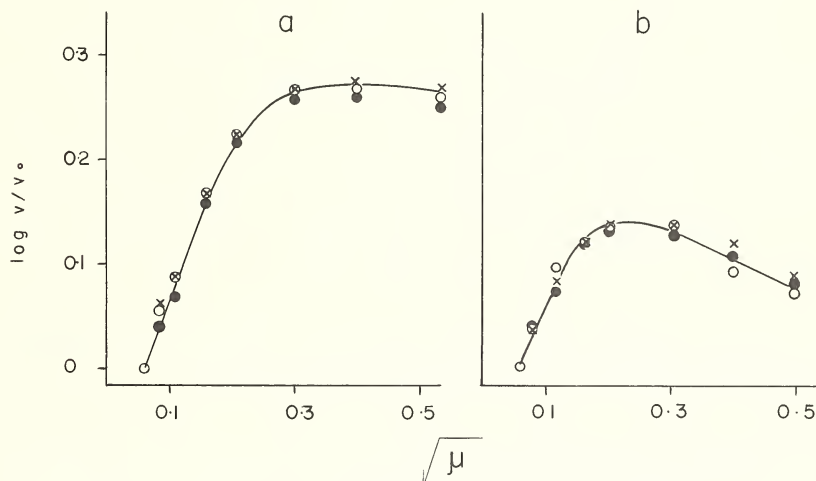
TEXT-FIGURE 2.—Effect of the dielectric constant on the system α -chymotrypsin-L-phenylalanine ethyl ester at 25 C in aqueous urea (a) and aqueous glycine (b) at pH 6.25 (●), 7.5 (○), and 8 (●).

teinase extracted from the latex of *Pileus mexicanus* (31, 32), characterized by being active in the absence of cysteine and EDTA (33). At pH 7.5 the α -chymotrypsin-catalyzed hydrolysis of either a specific substrate (PEE) or that of the trypsin substrate (BAEE) is accelerated continuously with the increase of ionic strength. Other salts produce similar effects (20, 21). At pH 6.25, the salt effect on the system α -chymotrypsin-PEE is less marked than at pH 7.5 and resembles that exerted on the system trypsin-BAEE. As with the dielectric constant effect, the difference between trypsin and chymotrypsin is evident only above pH 7. The salt effect on mexicain-BAEE is markedly enhancing at low values of ionic strength, but from $\sqrt{\mu} = 0.4$ onward the rate remains practically constant. Due to this fact, when the initial concentration of substrate is rather high, the salt effect cannot be detected. Even though less notable, something similar occurs with α -chymotrypsin: as the concentration of substrate is smaller, *e.g.*, with PEE, the system becomes more sensitive to small increments of ionic strength.



TEXT-FIGURE 3.—Effect of NaCl on the rates of hydrolysis catalyzed by: α -chymotrypsin at 25 C (a), trypsin at 25 C (b), and mexicain at 30 C (c). Curve aI, 0.016 M PEE at pH 6.25, curve aII, 0.016 M PEE at pH 7.5, curve aIII, 0.02 M BAEE at pH 7.5. Curve b, pH 7.5 0.008 M BAEE. Curve cI, 0.02 M BAEE, curve cII, 0.005 M BAEE, both cI and cII at pH 4.5.

Not only the esterolytic activity of proteinases is susceptible to changes in the medium which may alter electrostatic interactions; the proteolytic activity is also affected by an increase of the medium ionic strength. Text-figure 4 shows the effect of sodium, potassium, and lithium chlorides on the activity of α -chymotrypsin and trypsin on casein. The results resemble those obtained with ester substrates.

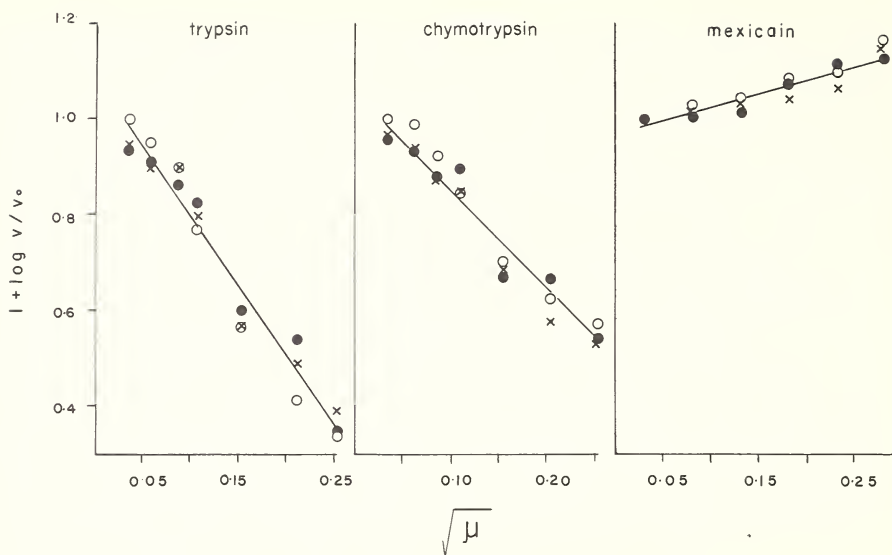


TEXT-FIGURE 4.—Effect of NaCl (●), KCl (○), and LiCl (x) on the rate of hydrolysis of casein (0.5% in NaOH 0.003 M) by α -chymotrypsin (a) and trypsin (b) at pH 7.5 and 35 C.

Ortega (33), in our laboratory, observed that the hydrolysis of urea-denatured hemoglobin by either trypsin or α -chymotrypsin is inhibited by an increase of ionic strength, while the mexicain-catalyzed hydrolysis of this substrate is slightly activated under these conditions (text-fig. 5). In the three cases there is a linear relationship between the rate logarithm and the square root of the ionic strength.

By viscosity measurements it was possible to ascertain the reason for the distinct results obtained with hemoglobin and casein. Urea affects more profoundly the conformation of chymotrypsin and trypsin than that of mexicain. Furthermore, an increase of ionic strength may reinforce the effect of urea (34). Apparently, as observed later (35), the conformational changes produced by urea in mexicain are propitious to the enzyme activity. Even though in the present case it seems that in order for salts to modify the protein conformation the prior action of urea is required, there are reports indicating that neutral salts by themselves are capable of bringing forth such effect (36, 37).

It has been proposed that formation of an acyl-enzyme intermediate compound, which is further decomposed in the presence of a nucleo-

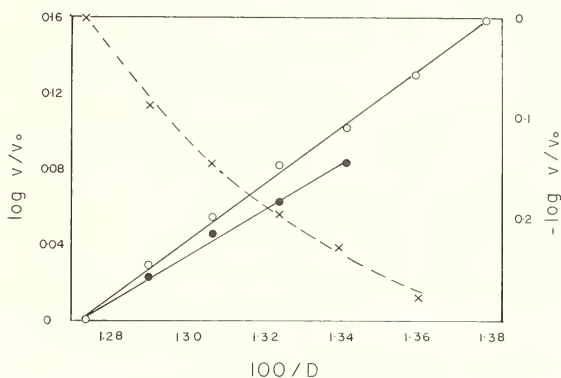


TEXT-FIGURE 5.—Effect of NaCl (●), KCl (○), and LiCl (x) on the rate of hydrolysis of hemoglobin (1% in 35% urea) at pH 7.5 and 35 C.

phile, is a possible pathway of reactions catalyzed by proteolytic enzymes (11). The use of a labeled substrate, acetyl-L-phenylalanine methyl ^{14}C -ester, made it possible to show that in the α -chymotrypsin-catalyzed hydrolysis of this ester in aqueous methanol, both water and alcohol compete as acceptors of the acyl group (38). Likewise, in the trypsin-catalyzed hydrolysis of L-lysine methyl ester (LME) at pH 6.2 in aqueous solutions of various alcohols, the respective products of transesterification, *i.e.*, L-lysine ethyl, propyl, butyl, and hydroxyethyl esters, could be identified in the hydrolysates. Accordingly, the liberation of titratable acid was slower (39). Nonetheless, in a recent study at this laboratory (28), the use of acetone (an aprotic solvent) made it possible to discern the rate dependence of the trypsin-catalyzed hydrolysis of LME on the macroscopic dielectric constant of the medium: this is similar to that previously observed with BAEE or TAME. On the other hand, the effect of *n*-propanol is converted from inhibitory to enhancing as pH increases, passing through a transition at pH 8.

Text-figure 6 shows the effect of acetone at pH 6.2 and that of *n*-propanol at pH 6.2 and 9. At the first pH value only acetone presents the pattern formerly observed in trypsin catalysis, namely, the already mentioned linear relationship in its positive slope of rate log and $1/D$. The effect of *n*-propanol is of the opposite trend and nonlinear. The transesterification product, L-lysine propyl ester was present in the hydrolysates (28). However, at pH 9 the effect of *n*-propanol has be-

come the same type as that which acetone exerts on the trypsin-catalyzed hydrolysis of LME at pH 6.2 or as those observed with alcohols on the hydrolyses of the *N*-acylated substrates BAEE or TAME (15, 28, 29). It seems that the relative predominance of the transesterification reaction over the dielectric constant effect has some relation to the ionization of the α -ammonium group of the substrate.



TEXT-FIGURE 6.—Effect of acetone (●) and *n*-propanol (x) at pH 6.2, and of *n*-propanol at pH 9 (○) on the trypsin-catalyzed hydrolysis of L-lysine methyl ester at 25 C as a function of the macroscopic dielectric constant.

CONCLUSIONS

While there is enough evidence indicating the solvent influence on the rate of enzymic reactions through the dielectric constant and/or the ionic strength, the mechanism by which this is achieved is not clear-cut. There is even much to clarify in the relation of these effects on chemical reactions (40). Various possible ways of modifying the reaction rate can be suggested, *viz.*:

- 1) through electrostatic interactions between charge(s) or dipole(s) in the active center of the enzyme and the substrate.
- 2) by modification of electrostatic interactions exerted by charges outside the active center, which presumably would be more important in the case of the protein substrates.
- 3) by change of the dissociation of ionizable groups.
- 4) by structural effects also dependent on the dielectric constant or the ionic strength.

If the interactions depicted in 1) were the only ones which operated, or at least the predominant ones, equations valid for chemical reactions such as those of Amis (3) would probably suffice to account for the observed effects.

On the other hand, Hiromi's treatment (26) taking into account the whole charge distribution of the enzyme molecule may be more adequate for proteolytic action. For a complete picture, however, effects 1 to 4 should be known. In addition to these, some other aspects of solvent effects must be considered, *e.g.*, the possibility that the microscopic dielectric constant or ionic strength near the reacting groups is different from the corresponding values of the bulk solution; the existence of selective solvation, or other specific effects such as transesterification, ion binding, etc.

The effect of amino acids and metabolites such as urea, which modify the dielectric constant, on the one hand, and salt effects on the other, might constitute a mechanism of activation and inhibition of enzymes with a greater or lesser significance in metabolic regulation.

RESUMEN

Se ha observado que la constante dieléctrica (D) y la fuerza iónica (μ) del solvente influyen sobre la velocidad de las reacciones catalizadas por tripsina, α -quimotripsina y otras proteinasas. Por encima de pH 7, el efecto de D y μ sobre la esterólisis catalizada por la tripsina o la α -quimotripsina es diferente. El efecto de μ sobre la esterólisis catalizada por la proteinasa vegetal mexicana es parecido al ejercido sobre sistemas en los que participa la α -quimotripsina. En la esterólisis catalizada por tripsina, la presencia de grupos α -amonio libres en el sustrato hace que, a pH menores de su pK_a , la reacción de transesterificación entre el sustrato y el alcohol predomine sobre el efecto dieléctrico del último con lo que ocurre una inhibición aparente. Sin embargo, la influencia verdadera de D puede apreciarse al emplear un solvente aprótico como la acetona.

La hidrólisis de la caseína catalizada por tripsina y α -quimotripsina se efectúa de manera parecida a la de sus contrapartes respectivas de la esterólisis, por lo que se refiere al efecto de μ . El efecto de μ sobre la proteólisis de la hemoglobina desnaturalizada con urea es distinto del de la hidrólisis de la caseína. Aparentemente, las sales neutras aumentan el efecto desnaturalizante de la urea, tanto en la hemoglobina como en las proteinasas. Como la tripsina y la α -quimotripsina son muy sensibles a la urea, su actividad proteolítica disminuye a medida que μ aumenta. La actividad proteolítica de la mexicana aumenta en presencia de la urea y con un aumento de μ .

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Factors Influencing the Activity of Membrane-Bound Enzymes in the Erythrocyte^{1,2,3}

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SUMMARY

Studies designed to obtain methodology to determine factors that interact with membranes and their influence on the behavior of membrane-bound enzymes have been reviewed. Of the three enzymes studied, the NAD nucleosidase and the acetylcholinesterase are firmly bound to the membrane because the totality of their activity is recovered in the ghosts. Their orientation appears to be external, since they act on nonpenetrating substrates and are attacked by trypsin present in the external medium. The case of the acid phosphatase is not as clear. While the enzyme in the intact erythrocyte acts on an external nonpenetrating substrate, only 60% of this activity can be recovered in the ghosts and during the hypotonic hemolysis required for the preparation of the ghosts, as soluble enzyme with similar specificity and pH optimum becomes apparent. It seems unlikely that the enzyme retained in

the ghosts is a contaminant of the soluble enzyme since the activity is present in the intact cell. It is likely, however, that the location of this enzyme in the membrane differs from that of the other two enzymes studied. Thus it is unaffected by trypsin and is not liberated by hypertonic KCl treatment, in contrast with the other two enzymes studied. The use of detergents at concentrations increasing gradually leads to interesting results that might clarify the manner in which the enzymes are interacting with the lipids of the membrane. Thus by means of ionic detergents, the surface charge may be modified and its effect on the enzymes detected. Since incorporation into a large detergent micelle appears to occur, rather than actual solubilization of the membrane components, this micellar-enzyme interaction may be profitably studied.—*Nat Cancer Inst Monogr* 27: 153-164, 1967.

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. J. P. Changeux, p. 165.

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THE MEMBRANE plays an important role in the regulatory mechanisms of the cell. Being a boundary between two solutions, it regulates the distribution of solutes between them. In addition, many of the cellular enzymes are membrane-bound. It is likely, therefore, that the activity of these enzymes may be controlled by factors which interact with the membrane *per se*.

An enzyme bound to the membrane may therefore be directly affected by the presence of an added substance or indirectly through modification of the original enzyme-membrane interaction by this substance. Thus, by simultaneous study of several enzymes bound to a membrane, specific effects will be those that inhibit or activate one enzyme without modifying the others, whereas a modifier of a membrane could lead to parallel changes in the activity of the various enzymes under investigation. The above premises are not mutually exclusive; however, their attractiveness is that they might permit the study of factors interacting with membranes by an approach independent of the usual measurement of transport rates.

This report summarizes studies on several enzymes that are bound to the rabbit erythrocyte membrane and the factors that can modify their activity owing to their special location in the cell.

LOCATION OF THE ENZYMES IN THE CELL

Three enzymes have been studied: acetylcholinesterase (acetylcholine acetyl-hydrolase 3.1.1.7), NAD nucleosidase (NAD glycohydrolase 3.2.2.5), and an acid phosphatase (orthophosphoric monoester phosphohydrolase 3.1.3.2.).

Intact rabbit erythrocytes suspended in isotonic saline are capable of hydrolyzing added NAD (1, 2), acetylcholine (2, 3), or *p*-nitrophenylphosphate (2), which indicates that the substrates can penetrate into the cell's interior or that the enzymes are present on the surface of the cell and can hydrolyze external substrates. During hypotonic hemolysis, the permeability of the cell increases markedly so that an equilibrium is reached between components in the interior and the exterior of the cell. Under these conditions, the rate of NAD (1, 2) and acetylcholine (2, 3) hydrolysis does not increase over that of the intact cell. However, the rate of *p*-nitrophenylphosphate hydrolysis rises to more than twice that of the intact cell (2). Since during hypotonic hemolysis, equilibrium is established across the membrane of the cell, it is possible by repeating this procedure under controlled conditions of ionic strength and *pH* (4) to obtain the membrane ("ghost") of the cell free of hemoglobin and of most internal soluble enzymes (5, 6). In table 1 we find that all of the NAD nucleosidase and acetylcholine esterase of the intact erythrocyte can be recovered in the hemoglobin-

TABLE 1.—Activity of NAD nucleosidase, acetylcholinesterase, and acid phosphatase in intact erythrocytes and in hemoglobin-free "ghosts"

Preparation	NAD nucleosidase		Acetylcholinesterase		Acid phosphatase	
	Units*/mg cholesterol	Units/mg protein	Units/mg cholesterol	Units/mg protein	Units/mg cholesterol	Units/mg protein
Erythrocytes.....	100	1	100	1	100	1
"Ghosts",	97.8 (93.4–105.8)	49.0 (46.6–52.9)	101.5 (90.1–109.7)	50.4 (44.6–55.0)	58.6 (57.7–62.0)	29.4 (28.6–30.4)

*A unit is defined as the amount of enzyme that hydrolyzes 1 μ mole of substrate per ml per minute. The assay procedures are those in (2). Ghosts were prepared essentially by the procedure of Dodge *et al.* (4). Cholesterol was determined by the method of Mårtenssen (7). Six independent determinations per value were performed.

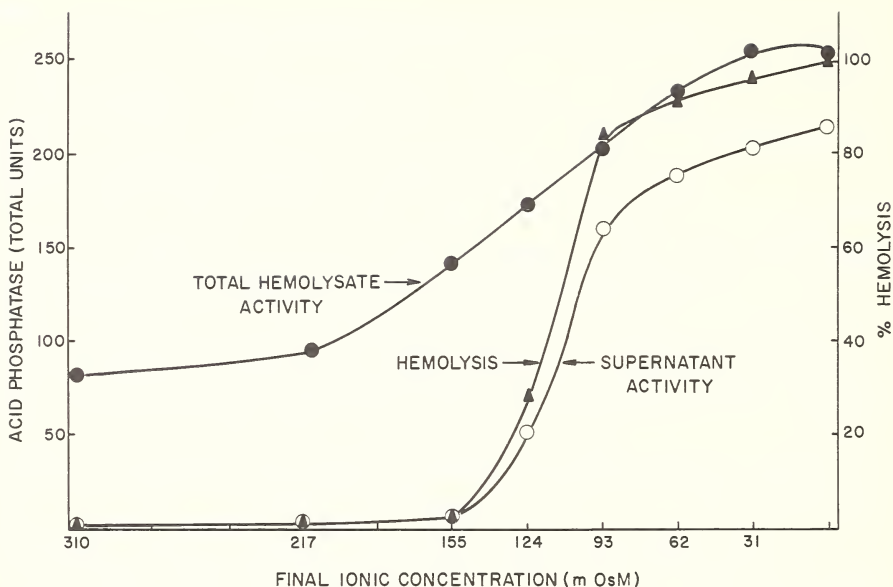
free ghosts, whereas only some 60% of the acid phosphatase is retained consistently.

The determinations shown in table 1 were performed under conditions in which the final ionic concentrations in the assay systems for both erythrocytes and ghosts were isotonic with the erythrocyte. Hemolysis was determined at the end of the assay period, and those systems showing more than 1% hemolysis were discarded. These precautions are critical in the case of the acid phosphatase since, as mentioned above and as shown in text-figure 1, hemolysis liberates an internal enzyme that can act on *p*-nitrophenylphosphate.

From the above results it is apparent that the NAD nucleosidase and the acetylcholinesterase are bound to the membrane, and probably all of the enzyme molecules are accessible to the external medium of the cell. In the case of the acid phosphatase, the situation is complicated by the presence of a soluble cytoplasmic enzyme in addition to that associated with the membrane. Thus, in the intact erythrocyte about 30–35% of the total hemolysate activity is detected, which indicates that the substrate is not permeable to the interior of the cell and that it reaches only a fraction of the total enzyme—the fraction conceivably bound to the membrane. However, when ghosts are prepared, these consistently contain some 60% of the activity detected in the intact erythrocyte. This fraction bound to the ghost is not removed by repeated washing of the ghosts in low ionic strength solutions.

BEHAVIOR OF ENZYMES IN THE ERYTHROCYTE AND IN THE GHOSTS

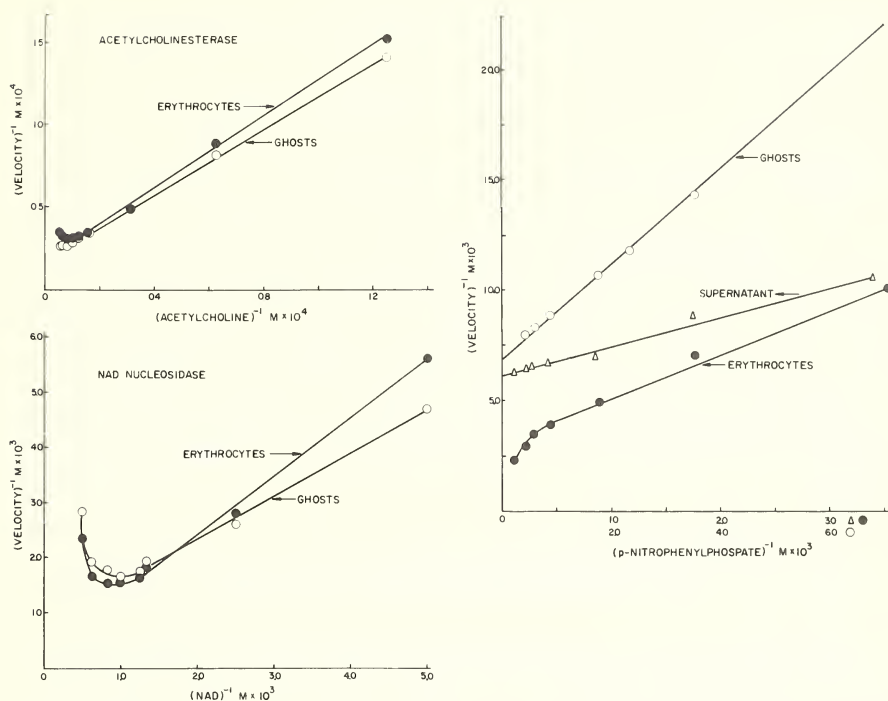
Hemoglobin-free ghosts, when placed in saline solutions, function as osmometers, which indicates that they have regained some of the permeability properties of the intact erythrocyte (6, 8). It therefore



TEXT-FIGURE 1.—Effect of hypotonic hemolysis on the activity of an acid phosphatase acting on *p*-nitrophenylphosphate. Rabbit red blood cells were incubated at room temperature at the final ionic concentrations shown. The activity of the acid phosphatase was assayed (2) in a system adjusted to 310 m Osm ionic concentration. The total suspension and the supernatant after centrifugation at $18,000 \times g$ for 25 minutes were studied.

appeared of interest to establish whether the kinetic behavior of the membrane enzymes was similar in the intact red blood cell and in the ghosts. Text-figure 2 shows as reciprocal plots the effect of substrate concentration on reaction velocity for the three enzymes presented. The assay systems were again isotonic with respect to the erythrocyte, and if hemolysis was detected the values were discarded. At high substrate concentrations, the rate of acetylcholine hydrolysis decreased both in the erythrocytes and in the ghosts. Although the response in both cellular suspensions was similar, it was observed in the case of the ghosts that the degree of inhibition varied markedly with the preparation being studied.

With NAD nucleosidase (text-fig. 2), a marked inhibition was again observed at high substrate concentrations, the behavior being quite similar in both cellular suspensions, but the response varied between different preparations. In the study of acid phosphatase, the results from tests with soluble cytoplasmic enzyme (supernatant) were also included. In this study the behavior of the three enzymes is quite different, although it should be indicated that in some preparations the downward slope observed in the erythrocytes at high substrate



TEXT-FIGURE 2.—Effect of substrate concentrations on the rate of hydrolysis catalyzed by enzymes of the intact erythrocyte and of hemoglobin-free ghosts. For conditions of the assays see (2).

concentrations could also be observed in the ghosts but not in the soluble enzyme.

Although these results are preliminary and designed only to contrast the behavior of the enzymes in the different preparations, it is probable that the inhibition observed with the acetylcholinesterase is due to excess substrate, as shown by Wilson (9), whereas that of the NAD nucleosidase is due to nicotinamide, as reported by Zatman *et al.* (10, 11). These aspects are presently under study.

Another comparison of the location of the enzymes in both preparations was gained from the study of the ability of the enzymes to attack by trypsin. Again, isotonic conditions were maintained in the assay systems to minimize hemolysis and decrease the penetrations of trypsin into the erythrocyte and into the ghost. In table 2 we note that the NAD nucleosidase is very liable to attack by trypsin, whereas the acid phosphatase is highly resistant, both in erythrocytes and in the ghosts. The acetylcholinesterase activity can be seen to decrease in both preparations, but that of the ghosts appears more readily accessible to the action of trypsin [see (12, 13)].

METABOLIC REGULATION

TABLE 2.—Effect of incubation with trypsin on the activity of membrane-bound enzymes in the rabbit erythrocyte*

Trypsin incubation (min)	Activity remaining after trypsin (%)					
	Acetylcholinesterase		Acid phosphatase		NAD nucleosidase	
	Erythrocytes	Ghosts	Erythrocytes	Ghosts	Erythrocytes	Ghosts
0.....	100	100	100	100	100	100
15.....	97.2	50.0	100	100	35	14.5
30.....	90.2	42.2	98.0	93.3	0	7.2
60.....	83.2	32.2	93.0	89.7	0	0

*Erythrocytes and ghosts were incubated with crystalline trypsin (1 mg/ml of the final suspension), and the reaction was stopped by the addition of 1 mg of egg-white trypsin inhibitor. The zero time control contained the trypsin inhibitor prior to the addition of the trypsin. Incubations and assays were performed under isotonic conditions.

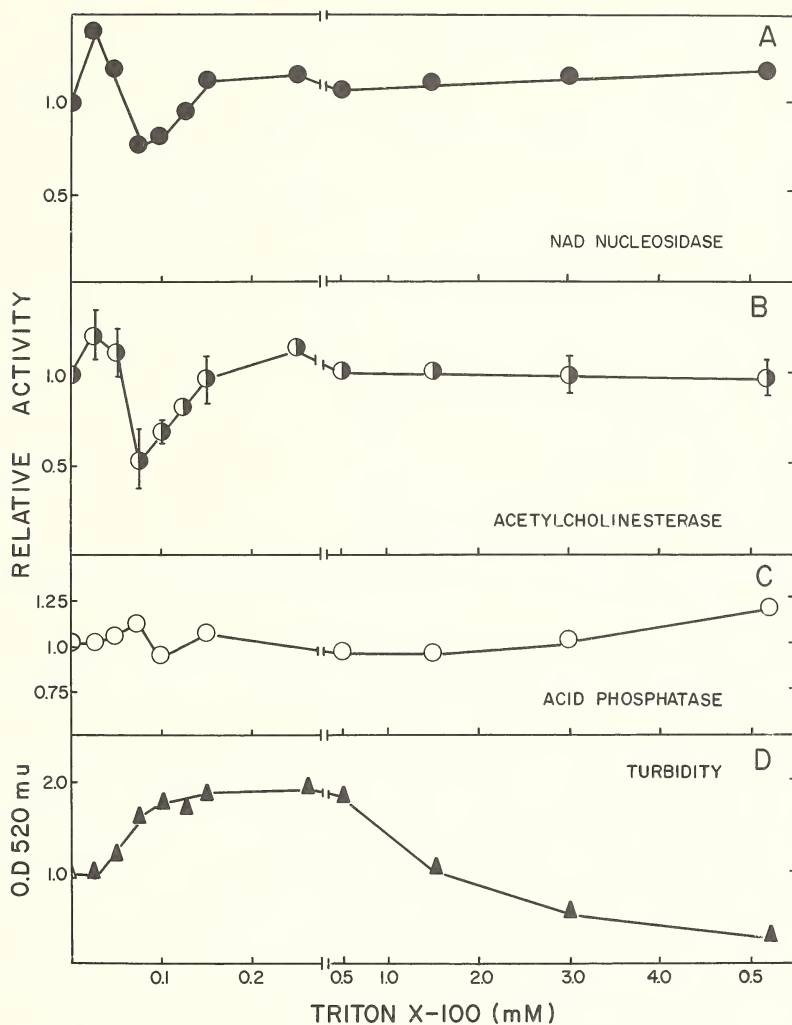
EFFECT OF DETERGENTS ON THE ACTIVITY OF MEMBRANE-BOUND ENZYMES

The addition of low concentrations of sodium lauryl sulfate (NaLS) or of cetyltrimethylammonium bromide (CTAB), labeled with radioactive isotopes, to ghost suspensions results in the binding of the majority of the detergent by the membrane of the cell (14). Triton X-100, a nonionic detergent, does not seem to incorporate into the membrane but appears to solubilize components from the membrane into the detergent micelle (14). Text-figures 3, 4, and 5 show the effect of increasing concentrations of the above detergents on the activity of the membrane enzymes under study. Low concentrations of Triton X-100 (text-fig. 3) lead to a slight activation of the three enzymes; however, further addition of the detergent causes a rise in the turbidity of the suspension accompanied by an inhibition of the three enzymes, which is most accentuated with acetylcholinesterase. This inhibition of the enzymes disappears at higher detergent concentrations, and the enzymes remain active even at those detergent concentrations where complete "solubilization" of the membrane is observed.

When CTAB is added at low concentrations to a ghost suspension (text-fig. 4), a rapid inhibition of the acetylcholinesterase results, whereas the NAD nucleosidase is inhibited only when complete "solubilization" of the suspension occurs. The acid phosphatase is not inhibited by any of the CTAB concentrations studied.

The addition of NaLS inhibits the activity of the three enzymes (text-fig. 5) only at concentrations where "solubilization" occurs.

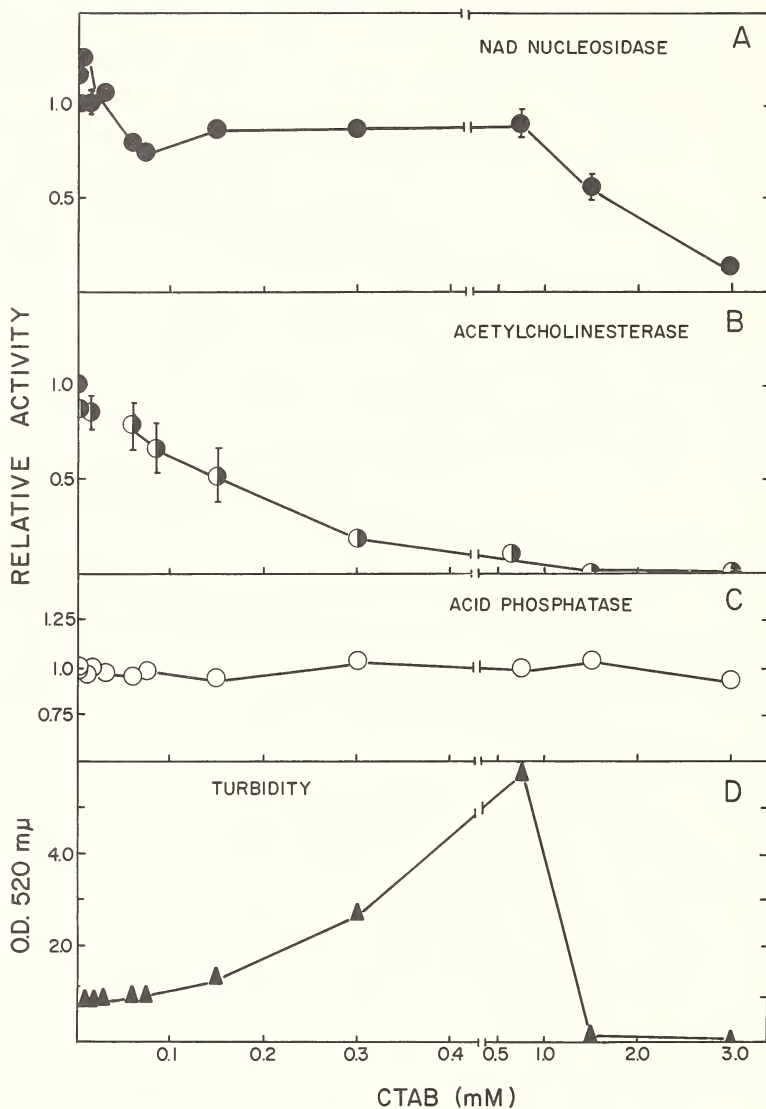
Dialysis of the "solubilized" suspensions to remove the detergents results in a reactivation of the enzymes when CTAB is used, but not



TEXT-FIGURE 3.—Effect of Triton X-100 on the turbidity of ghost suspensions and on the activity of three membrane-bound enzymes.

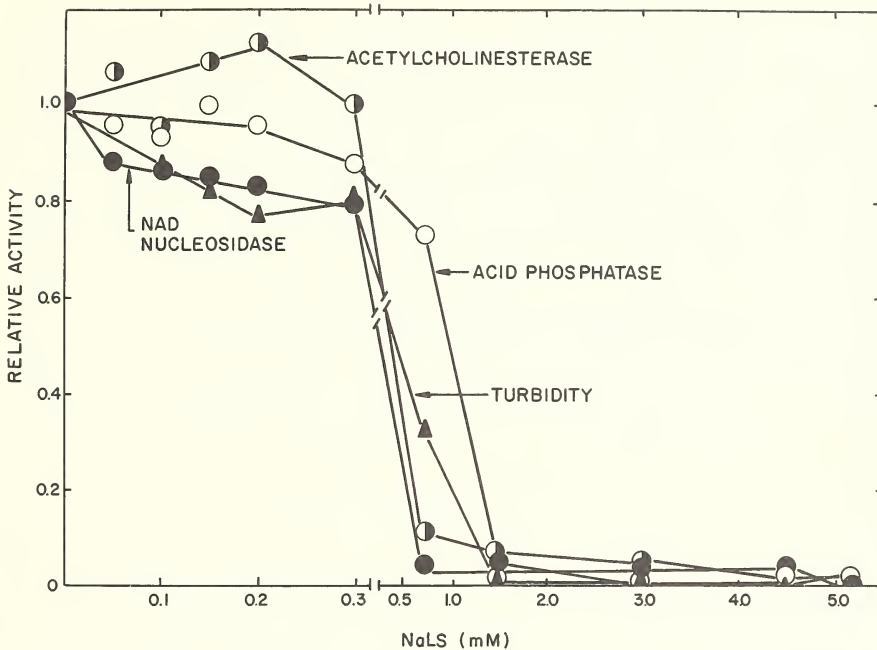
when NaLS is used. Most of the membrane components that cannot be sedimented at $105,000 \times g$ for 60 minutes when “solubilized” readily sediment when the detergents have been removed.

The above results have been interpreted (14) on the basis of a mixed micelle being formed between the added detergents and the phospholipids of the ghosts, by direct incorporation of the ionic detergents into the membrane, and by solubilization of membrane components in the case of the nonionic detergent.



TEXT-FIGURE 4.—Effect of cetyltrimethylammonium bromide (CTAB) on the turbidity of ghost suspensions and on the activity of three membrane-bound enzymes.

The interactions of the detergent with the enzymes could therefore be due to two types of effects: first, to a modification by the detergent of the orientation and charge of the membrane or, second, to the direct interaction of the detergent with the enzyme. The inhibition of the three enzymes by Triton X-100 appears to be of the first type and the inhibition of acetylcholinesterase by CTAB of the second type. It is



TEXT-FIGURE 5.—Effect of sodium lauryl sulfate (NaLS) on the turbidity of ghost suspensions and on the activity of three membrane-bound enzymes.

known that quaternary ions can inhibit acetylcholinesterase (9). The fact that the “solubilized” enzymes become sedimentable upon removal of the detergent indicates that the enzymes are not in true solution but are associated with the mixed micelles, which are nonsedimentable under the conditions used because of the excess of detergent.

In relation to the above, it is of interest that treatment of the ghosts with phospholipase C, which converts nearly all of the phospholipids of the membrane to diacylglycerol plus the phosphoryl base or amino acid, does not alter the activity of the three enzymes. Taking advantage of this result, an acetone powder nearly free of phospholipids was prepared after phospholipase C treatment. This powder in suspension has all the enzymatic activity of the original ghosts. It is being used in attempts to prepare the enzymes in a soluble form free of lipids.

FRAGMENTATION OF THE GHOST MEMBRANES WITH HYPERTONIC SALT SOLUTIONS

If hemoglobin-free ghosts are placed in hypertonic solutions of salts, a marked fragmentation of the membranes results (15, 16). The effect is related to the nature of the anion present in the salt. Thus, $KCl < KI$

<KSCN (16). If the excess salt is removed after salt treatment and the membrane fragments are sedimented in a linear sucrose gradient, two bands are obtained. Band 1 is a light polydisperse band with an average density of 1.145, and band 2 is a heavier, more compact band with an average density of 1.185. If intact ghosts are spun in a similar gradient, only band 2 is formed. Table 3 shows the distribution of membrane components and of the three enzymes studied in the bands generated in the sucrose gradient after treatment of the ghosts with KCl and KI.

TABLE 3.—Salt fragmentation of rabbit erythrocyte ghosts*

Membrane component	Salt treatment			
	0.8 M KCl		0.8 M KI	
	Band 1	Band 2	Band 1	Band 2
	(% of the total recovered)			
Phospholipid phosphorous.....	30	70	83	17
Protein.....	17	83	63	37
Carbohydrate.....	42	58	76	24
NAD nucleosidase.....	34	66	80	20
Acetylcholinesterase.....	45	55	75	25
Acid phosphatase.....	4	96	62	38

*The ghosts were exposed to the hypertonic salt for 1 hour. The procedure is that of Martínez-Zedillo *et al.* (16).

Treatment with 0.8 M KCl results in the liberation of fragments containing a higher lipid and carbohydrate to protein ratio than the original ghosts. These fragments contain the NAD nucleosidase and acetylcholinesterase but almost none of the acid phosphatase. The KI treatment is more drastic and the fragments formed now also contain the acid phosphatase, although a greater proportion of lipid, carbohydrate, NAD nucleosidase, and acetylcholinesterase over that of protein and acid phosphatase is again observed.

RESUMEN

Se presenta una revisión de los estudios planeados para tener métodos que permitan determinar los factores que interaccionan con las membranas y su influencia sobre las enzimas unidas a membranas.

De las tres enzimas estudiadas, la NAD nucleosidasa y la acetil colinesterasa están firmemente unidas a la membrana pues la totalidad de su actividad se encuentra en los fantasmas de glóbulos rojos. Su orientación parece ser externa puesto que actúan sobre sustratos no penetrantes y son atacadas por la tripsina presente en el medio externo. El caso de la fosfatasa ácida no es muy claro; aunque

la enzima de los eritrocitos intactos actúa sobre sustratos externos no penetrantes, sólo se recupera el 60% de dicha actividad en los fantasmas; además, durante la preparación de los fantasmas, con la hemólisis hipotánica, se hace ostensible una enzima soluble con especificidad y *pH* óptimo parecidos. Es poco probable que la enzima retenida en los fantasmas sea un contaminante de la enzima soluble puesto que la actividad está presente en la célula intacta. Sin embargo, es probable que la localización de esta enzima en la membrana sea diferente a la de las otras dos enzimas estudiadas. Así, no es afectada por la tripsina y no es liberada con el tratamiento de KCl hipertónico, en contraste con las otras dos enzimas estudiadas.

El empleo de detergentes a concentraciones progresivamente crecientes da resultados que pueden aclarar el modo como las enzimas inactúan con los lípidos de las membranas. Así, por medio de detergentes iónicos, es posible modificar la carga de superficie y reconocer su efecto sobre las enzimas. Como parece ocurrir su incorporación en una gran micela de detergente, más que la solubilización real de los componentes de la membrana, esta interacción micela-enzima puede ser estudiada con grandes ventajas.

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DISCUSSION ¹

Discussor, DR. J. P. CHANGEUX, Department of Molecular Biology, University of California, Berkeley, California

THE discussion was started with a short comment by J. P. Changeux (Virus Laboratory, University of California, Berkeley), on the model of allosteric interactions recently proposed by Monod, Wyman, and Changeux (1965). A basic postulate of this model, which distinguishes it from the model of Koshland, Nemethy, and Filmer (1966), is that the conformational transition of an allosteric protein is an all-or-none phenomenon involving simultaneous (but not sequential) change of the structure of the several subunits of the molecule.

This hypothesis constitutes, in fact, an attempt to correlate the cooperativity of ligand binding, observed with a large number of regulatory proteins, with the cooperativity of structure of the protein molecule. All the regulatory proteins, which have been studied in detail, are made of several identical subunits or protomers (oligomeric state). They thus fall into the category of *ordered* structures. Dr. Changeux then extended the analogy of the oligomeric molecules with other ordered structures made of the cooperative association of repeating units: crystals or solids. Presence of symmetry is the simplest element which characterizes their order. In this context the "concerted" transitions of an oligomeric molecule can be understood as a microscopic "phase transition" or a framework rearrangement analogous to those observed between "allotropic" forms of a crystal which preserve the symmetry of the structure.² Obviously the scale is different and the proteins considered are globular and thus "closed" or *limited* structures.

Presence of a fraction of "hybrid" molecules, where the protomers are simultaneously present under *different* conformations (a basic

¹ Of articles by Victoria Chagoya, Alberto Hamabata, and José Laguna; D. E. Koshland, Jr., and M. E. Kirtley; Luz María del Castillo, and M. Castañeda-Agulló; and Carlos Gitler, Gustavo Martínez-Zedillo, Dalila Martínez-Rojas, and Guadalupe Chavez-Díaz.

² See Lipscomb, Science 153: 373, 1966.

principle of Dr. Koshland's model), is not completely excluded. The problem is whether this fraction can be neglected or not. The presently available data obtained with glyceraldehyde-3-phosphate dehydrogenase by Kirschner, Eigen, Bittman, and Voigt³ and with aspartate transcarbamylase (Changeux, Gerhart, Rubin, and Schachman, manuscript in preparation) suggest that in *first approximation*, the assumption of Monod, Wyman, and Changeux that the *symmetrical* states of the allosteric molecules are the more stable is valid with these systems.

Another comment on Dr. Koshland's paper was presented by Dr. Osvaldo Cori (Cátedra de Bioquímica general-Facultad de Química y Farmacia-Universidad de Chile) on some aspects of the substrate specificity of potato apyrase (ATP diphosphohydrolase, 3.6.1.5.) obtained in his laboratory. This enzyme splits pyrophosphate bonds, but only if one extreme of the molecule contains an organic group, and the other end is free. This, as well as inhibition of ADP hydrolysis by triphosphates, can be simply explained in terms of the induced fit hypothesis.

Substrate	Relative V_{max}
ATP.....	100
Other nucleotide triphosphates (deoxy ATP, UTP, ITP, CTP).....	50-85
Benzyl triphosphate.....	85
Phenylpropyltriphosphate.....	55
ADP.....	20
Benzyl diphosphate.....	18
AMP.....	0
Inorganic pyrophosphate, inorganic tripolyphosphate.....	0
NAD ⁺ , ADP-glucose.....	0
Diadenosine triphosphate or tetraphosphate.....	0
ATP methylester.....	0
Adenosine tetraphosphate.....	5

Furthermore, the organic triphosphates are powerful, competitive inhibitors of the hydrolysis of the diphosphates. The inorganic pyrophosphates are also inhibitors, but the NAD⁺ or ADP-glucose type molecules are neither substrates nor inhibitors. Dr. Cori thinks that it is not possible to explain these or the K_m data reported elsewhere by the template theory. (Some of these data are adapted from: O. Cori, A. Traverso-Cori, M. Tetas, and H. Chaimovich.)⁴

Finally, Dr. E. Fischer raised the important question of the dimension of the change of structure expected in a protein molecule upon binding of a stereospecific ligand. In the case of hemoglobin as shown by Perutz and co-workers, the structural change upon oxygen binding involves displacements of amino acid residues at distances of 7 or 8 Å. On the other hand, from the X-ray crystallographic data, Phillips and co-workers have demonstrated that binding of a substrate analogue to lysozyme changes the position of certain groups in the active site by

³ Proc Nat Acad Sci USA 56: 1001, 1966.

⁴ Substrate specificity and inhibition studies on potato apyrase. Biochem Z 342: 345, 1965.

only 0.7 Å. For the real substrate they do not expect a change of more than 1 Å.

Finally, Dr. Changeux emphasized that there might exist a difference of *scale* between the structural changes involved in 1) the allosteric interactions between distinct and distant sites (*e.g.*, hemoglobin) and 2) the interaction of the ligand with its own binding site or with a topographically close site. One may have to distinguish in the future between “macroscopic” and “microscopic” conformational alterations in protein molecules corresponding, respectively, to the former and to the latter type of interactions.

Protein Synthesis in Wheat Embryos^{1,2,3}

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SUMMARY

During germination, the synthesis of new proteins (enzymes) must be important in the mechanism whereby the plant seed passes from a latent to a highly active state. Thus we looked first at the protein-synthesizing machinery of ungerminated wheat embryos. The *in vitro* system obtained from such embryos is completely inactive in the incorporation of amino acids into proteins. However, the addition of synthetic messenger RNA in the form of polynucleotides causes a dramatic increment in amino acid incorporation. This property has been utilized in the investigation of the genetic code in wheat. Phenylalanine incorporation in the presence of polyuridylic acid and the ungerminated wheat embryo system requires ribosomal particles, supernatant fluid, soluble RNA (sRNA), adenosine triphosphate, guanosine triphosphate, a nucleoside triphosphate regenerating system, and magnesium and potassium ions. The isolated ribosomes are mostly in the form of monomers as determined by density gradient sedimentation analysis. Resedimented wheat embryo ribosomes

are completely dependent on supernatant enzymes for polyphenylalanine synthesis from phenylalanyl-sRNA. This aminoacyl "transfer" reaction requires also polyuridylic acid, guanosine triphosphate, Mg^{++} and K^+ ions and SH groups. The transfer enzymes can be partially purified by ammonium sulfate precipitation and diethylaminoethyl chromatography. The specificity of the ribosomes for the supernatant factor in the transfer reaction was studied with ribosomes from different species. The mammalian and wheat ribosomes could interchange their supernatant factors to give aminoacyl transfer but were not active with the *Escherichia coli* factor. *E. coli* ribosomes could only use the homologous supernatant fraction. The tobacco-leaf chloroplast ribosomes were active with the wheat supernatant fluid. sRNA isolated from both ungerminated and germinated embryos has been analyzed by methylated serum albumin chromatography. Of nine aminoacyl-sRNAs studied, only valyl and methionyl-sRNAs showed reproducible changes in the chromatographic profiles.—*Nat Cancer Inst Monogr* 27: 169-179, 1967.

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. M. P. Stulberg, p. 193.

³ This research was supported by project No. 164 of the Jane Coffin Childs Memorial Fund for Medical Research and by the University of Chile-University of California Program.

⁴ Presently a John Simon Guggenheim Foundation Fellow at the Laboratory of Biochemical Genetics, the National Heart Institute, National Institutes of Health, Bethesda, Md.

⁵ Maria Bravo, Marta Gatica, Guillermo Oestreicher, and German Zanghellini participated in these experiments. Some of the work was done in the laboratory of Dr. C. R. Stocking, Department of Botany, University of California, Davis, Calif., while I was a visiting scholar participating in the University of Chile-University of California Program. We are extremely grateful to Dr. Stocking for his encouragement and advice.

THE GERMINATION PROCESS converts a metabolically quiescent plant seed into a rapidly growing and differentiating tissue. The regulation of this transition must involve a trigger mechanism that activates the synthesis of proteins necessary for the new cellular functions. For this reason, it seemed interesting to look for differences in the protein-synthesizing machinery of seeds before and after germination.

So far, most of our studies have been done with ungerminated wheat embryos of a genetically pure strain of *Triticum durum*.

METHODS

Viable wheat embryos were isolated from the seed by the method of Johnston and Stern (1). Ribosomal particles and supernatant fraction were obtained from a total embryo homogenate by the usual method of differential centrifugation. Ribosomes for the aminoacyl transfer reaction were "washed" by resedimenting in the homogenization buffer. Ribosomes from *Escherichia coli* and guinea pig liver that were dependent on the supernatant fraction for aminoacyl transfer were prepared by the procedures of Nathans and Lipmann (2, 3). Tobacco-leaf chloroplast ribosomes that were predominantly of the 66 S variety were kindly supplied by Dr. C. R. Stocking.

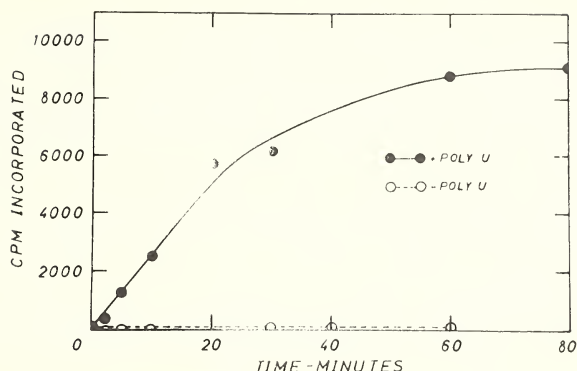
Soluble RNA (sRNA) from wheat embryos and rat liver was isolated by the method of Brunngraber (4), and ^{14}C -phenylalanyl-sRNA was prepared by the procedure of von Ehrenstein and Lipmann (5).

RESULTS

Text-figure 1 shows that very little incorporation of ^{14}C -phenylalanine takes place in the ungerminated wheat embryo system; the addition of polyuridylic acid (poly U), however, causes more than 100-fold stimulation of this incorporation. The low endogenous activity and the excellent response of this system to uracyl-containing polynucleotides permitted us, in collaboration with Dr. Basilio, to investigate for the first time the genetic code of a higher plant (6).

The properties of this poly U-stimulated system are very similar to those described for *E. coli* (8) and for many other systems from different sources. The activity is absolutely dependent on the presence of ribosomes, Mg^{++} , and ATP and its regenerating system. Definite requirements are also observed for GTP, wheat sRNA, supernatant fluid, and potassium ion (9). Soluble RNA from yeast or rat liver can replace the wheat sRNA.

Sucrose gradient sedimentation of the ribosomes used in these experiments showed that the particles were predominantly monomers



TEXT-FIGURE 1.—Time course of incorporation of ^{14}C -phenylalanine. Amino acid-incorporation mixtures of 10 ml, with components as previously described (6), were incubated at 37 C in the presence and absence of 1.25 mg of poly U. At the times indicated, 1 ml aliquots were pipetted into 5% trichloroacetic acid, and the amino acid incorporated into polypeptide was measured in a gas-flow counter with 39% efficiency according to the procedure of Zamecnik *et al.* (7).

with a sedimentation coefficient of 80 S. The absence of appreciable polysomal material is consistent with the low endogenous incorporating activity observed and agrees with the report for ungerminated peanut cotyledons (10).

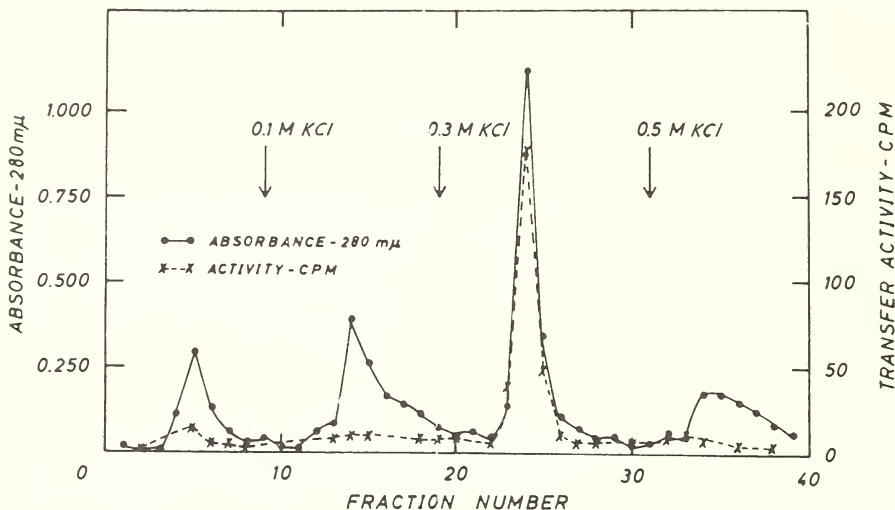
In other species, aminoacyl transfer from aminoacyl-sRNA to polypeptide has been shown to require the presence of several "transfer" enzymes in the supernatant fraction (11-13). Table 1 shows that just 1 ribosome wash is enough to make the wheat particles completely

TABLE 1.—Requirements for synthesis of polyphenylalanine in the wheat-embryo transfer system*

System	cpm transferred	Activity (%)
Complete.....	294	100
Without ribosomes.....	8	3
Without supernatant fraction.....	28	9
Without polyuridylic acid.....	14	5
Without magnesium.....	5	2
Without KCl.....	56	19
Without guanosine triphosphate.....	30	10
Without P-enolpyruvate.....	40	13
Without pyruvate kinase.....	87	29
Without P-enolpyruvate and pyruvate kinase.....	16	5

*The transfer reaction mixture contained in 1 ml 50 mM Tris-HCl, pH 7.5; 25 mM KCl; 7.5 mM MgCl_2 ; 0.5 mM guanosine triphosphate; 150 μg of RNA as once-washed ribosomes; 0.5 mg of protein of the supernatant fraction, 40 μg of polyuridylic acid; 5 mM P-enolpyruvate; 10 μg of pyruvate kinase; and ^{14}C -phenylalanyl-sRNA, containing approximately 1500 cpm of ^{14}C -phenylalanine (specific activity 100 $\mu\text{C}/\mu\text{mole}$). The mixture was incubated for 15 minutes at 37 C. The reaction was stopped with 5% trichloroacetic acid and polyphenylalanine synthesis was measured.

dependent on the supernatant fraction for polyphenylalanine synthesis from phenylalanyl-sRNA in the presence of poly U. As in other systems, the unknown series of reactions of the transfer process also require the addition of GTP and its generating system and of Mg^{++} and K^+ .



TEXT-FIGURE 2.—Diethylaminoethyl-cellulose chromatography of the wheat-embryo transfer factor. The high-speed supernatant fraction (20 ml) from a wheat embryo homogenate was precipitated with ammonium sulfate between 40 and 80% saturation. The precipitated protein (10 mg) was dissolved in 0.01 M Tris at pH 7.5, passed through a Sephadex G-50 column (10 × 1.2 cm), and applied to a diethylaminoethyl-cellulose column that had been equilibrated with 0.01 M Tris, pH 7.5. Stepwise elution was achieved with the same buffer containing initially no added salt and subsequently 0.1 M, 0.3 M, and 0.5 M KCl as indicated in the text-figure. The phenylalanyl transfer activity was measured for 50 μ l aliquots.

The transfer factor present in the supernatant can be partially purified by ammonium sulfate precipitation and DEAE-cellulose column chromatography as shown in text-figure 2.

The transfer enzyme obtained by this method is quite unstable even at low temperatures. We have some indications that this fraction might have several components, as has been found in other species, but as yet we have not been able to obtain a clear separation of them.

It is interesting that aminoacyl-sRNA binding to the ribosome-messenger complex of the type described by Nirenberg and Leder (14) occurs with the wheat system and does not require transfer enzymes or GTP (6). Recently we have observed that this binding is greatly inhibited by the tetracycline antibiotics as described in *E. coli* (15).

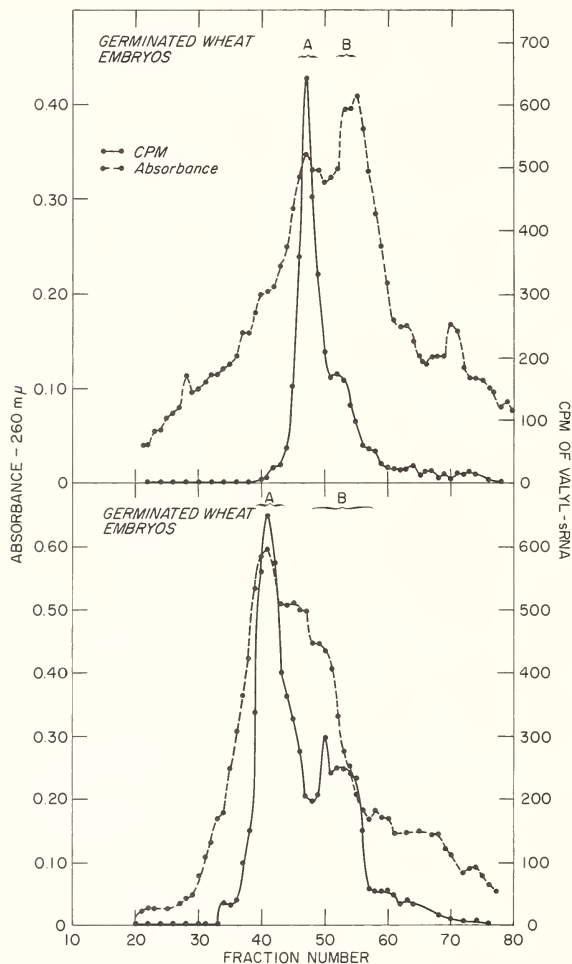
Table 2 shows some experiments that test the species specificity of the ribosomes and supernatant fractions from *E. coli*, guinea pig liver, wheat embryos, and tobacco-leaf chloroplast ribosomes. It is evident that the mammalian and the wheat system can interchange their components and maintain activity. The bacterial components, however, are not active in the mixed systems. Tobacco-leaf chloroplast ribosomes, which resemble the bacterial particles in their sedimentation coefficient (66 S), were active with the wheat supernatant. Unfortunately, we had a limited supply of these ribosomes and were not able to test them with other supernatants.

TABLE 2.—Species specificity of the transfer system

Ribosomes	Supernatant fraction	cpm transferred
Wheat embryo.....	None	10
<i>Escherichia coli</i>	None	14
Tobacco-leaf chloroplast.....	None	60
Guinea pig liver.....	None	15
None.....	Wheat embryo	18
None.....	<i>E. coli</i>	20
None.....	Guinea pig liver	3
Wheat embryo.....	Wheat embryo	634
Wheat embryo.....	<i>E. coli</i>	24
Wheat embryo.....	Guinea pig liver	306
<i>E. coli</i>	Wheat embryo	29
<i>E. coli</i>	<i>E. coli</i>	796
<i>E. coli</i>	Guinea pig liver	15
Guinea pig liver.....	Wheat embryo	709
Guinea pig liver.....	<i>E. coli</i>	23
Guinea pig liver.....	Guinea pig liver	440
Tobacco-leaf chloroplast.....	Wheat embryo	530

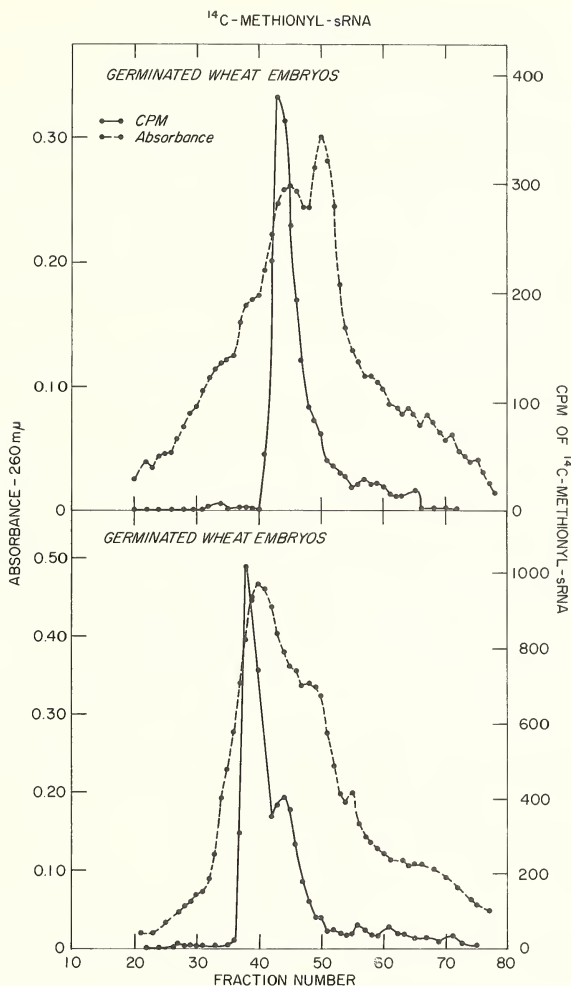
Recently we have been studying the methylated serum-albumin chromatography profiles of aminoacyl-sRNA isolated from wheat embryos before and after 4 days' germination. So far we have examined 9 amino acids: threonine, phenylalanine, leucine, serine, valine, methionine, aspartic acid, glutamic acid, and glycine. The method gives highly reproducible results indicating that there are differences in the bulk sRNA at the 2 stages of development (see 260 m μ absorbance profiles in text-figs. 3 and 4).

Some aminoacyl-sRNAs such as those specific for glycine, threonine, and glutamic acid showed no differences after seed germination. Others such as phenylalanine, leucine, serine, and aspartic acid showed greater heterogeneity before germination than after, but the differences were not very conclusive. Clear differences were apparent in methionine



TEXT-FIGURE 3.—Methylated serum albumin chromatography of ^{14}C -valyl-sRNA of wheat embryos before and after germination. ^{14}C -valyl-sRNAs from ungerminated (1.2 mg with 3600 cpm) and from 4-day-germinated wheat embryos (1.6 mg with 4500 cpm) were chromatographed on methylated serum-albumin columns according to Sueoka and Yamane (16). The fractions (2 ml) were read for absorbance and subsequently precipitated with 5% trichloroacetic acid, filtered on Millipore membranes, and counted in a gas-flow counter with 39% efficiency.

and valine (text-figs. 3 and 4). For valine, the ratio of peak B to A goes from 0.57 before germination to 0.32 in the germinated tissue. The second methionyl-sRNA peak almost disappears after germination.



TEXT-FIGURE 4.—Methylated serum albumin chromatography of ^{14}C -methionyl-sRNA of wheat embryos before and after germination. ^{14}C -methionyl-sRNA from ungerminated wheat embryos (1.0 mg with 2600 cpm) and 4-day-germinated wheat embryos (1.5 mg with 4400 cpm) were chromatographed and treated as described in text-figure 3.

DISCUSSION

It is apparent from the results described that the inactivity of the endogenous amino acid-incorporating system from ungerminated wheat embryos is not due to a major deficiency of its protein-synthesizing machinery. When poly U is added, the system is quite active and shows typical properties and requirements whether tested for

amino acid incorporation, aminoacyl transfer, or aminoacyl-sRNA binding to the ribosome-poly U complex. We could conclude, therefore, that what the system lacks is "active" messenger RNA. This conclusion would be in agreement with that reached for sea-urchin eggs prior to fertilization (17) and by Marcus and Feeley (18) for ungerminated peanut cotyledons. Recently, however, several workers (19-21) have described special factors that are required for natural messenger translation and are not required for poly U-directed phenylalanine incorporation. These factors could be altered in the ungerminated tissue.

It would be interesting to determine whether before germination there is an actual absence of messenger RNA or whether the messenger is in a "masked" form (22).

The species-specificity studies with ribosomes and transfer enzymes, together with similar reports for other species (2, 23-25), seem to point to a basic difference between the components of nucleated cells (yeast, insects, mammals, birds, wheat) and those of bacterial origin. This difference may be important in allowing these two types of cells to solve their diverse regulatory problems and may explain their different susceptibility to antibiotics such as chloramphenicol.

Ames and Hartman (26) have proposed a regulatory role for sRNA at the translation level of protein synthesis. It does not seem likely that this type of regulation constitutes the major trigger mechanism in germinating plant seeds since endogenous amino acid incorporation remains inactive when sRNA from rat liver or yeast is added, even though the polynucleotide-stimulated system can use the sRNA from these two species as well as the homologous component. It is quite possible, however, that variations in sRNA fractions accompany and modulate the radical changes in protein synthesis during the germination process. Examples of such changes have been reported in sporulating bacteria (27, 28). Valine- and methionine-specific sRNAs in wheat embryos seem to be other examples. Methionyl-sRNA, of course, has the added interest of being involved in the chain initiation process in bacterial *E. coli* protein synthesis (27). The methylated serum-albumin chromatographic method, although very useful in analytical studies, has the limitation of charge capacity, and thus it has not been possible to yet establish whether the differences found are due to configuration changes of the sRNA, to its interactions with other components, or whether they really represent relative changes in codon-specific fractions. Presently, we are employing the partition chromatography method of Kelmers, Novelli, and Stulberg (30) to further investigate these phenomena.

RESUMEN

Durante el proceso de la germinación, la biosíntesis de nuevas proteínas debe jugar un papel importante en el mecanismo por el cual la semilla pasa de un estado latente a uno de gran actividad.

Nosotros hemos investigado este proceso a través de un estudio de la maquinaria para la biosíntesis proteica en los embriones de trigo antes de germinar. El sistema *in vitro* que se obtiene de ese material es totalmente inactivo en la incorporación de amino ácidos en proteínas. Sin embargo, la suplementación de dicho sistema con RNA mensajero sintético en forma de polinucleótidos, resulta en una pronunciada estimulación de la incorporación de amino ácidos. Esta capacidad se ha utilizado en el estudio del código genético en trigo. Incorporación de fenilalanina en el sistema de embrión de trigo sin germinar requiere, además de ácido poliuridílico, partículas ribosomales, fracción sobrenadante, RNA soluble, ATP, GTP, un sistema regenerador de nucleosido-trifosfatos y iones magnesio y potasio. Las ribosomas aisladas están mayormente en forma de monómeros según se puede determinar en sedimentación con gradiente de densidad.

La síntesis de polifenilalanina a partir de fenilalanil-sRNA requiere la presencia de un factor enzimático del sobrenadante siempre que las ribosomas se limpien por resedimentación. Por medio de precipitación con sulfato de amonio y cromatografía se ha logrado purificar parcialmente esta enzima de transferencia. Los sistemas de trigo, hígado de conejo y cloroplastos de hoja de tabaco pueden intercambiar entre sí ribosomas y enzimas de transferencia, pero los componentes de *Escherichia coli* solo son activos en un sistema homólogo.

Análisis cromatográfico de nueve aminoacil-sRNA de germen de trigo antes y después de la germinación, muestra diferencias en los valil y metionil-sRNA de los dos estados de desarrollo.

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RNA Code Words in Several Species^{1,2,3}

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SUMMARY

Systems incorporating amino acids have been prepared from chick embryo and wheat germ. Both systems responded to synthetic polynucleotides, and both had absolute requirement for energy and for magnesium ions. Puromycin and ribonuclease inhibited the polynucleotide-stimulated incorporation of the amino acids, and sulfhydryl compounds were not essential. GTP appeared to be required in wheat germ, but it had no effect in chick embryo. Wheat germ was dependent on sRNA, whereas chick embryo was inhibited by the extra addition of this component. Codon assignment for the different amino acids was made in chick embryo and wheat germ systems, with syn-

thetic polynucleotides of known composition as artificial messengers. The codons found in both systems coincided almost perfectly with those previously reported for *Escherichia coli*, except that the 2UIC codon found in *E. coli* and chick embryo was not present in wheat germ. The significance of this finding is discussed in relation to a possible mechanism controlling the synthesis of proteins. An explanation for the universality of the genetic code is discussed on the basis of the almost absolute improbable changes that should occur in the genetic material to change the code.—Nat Cancer Inst Monogr 27: 181-192, 1967.

THE PRINCIPLE of the universality of the genetic code has been postulated on the basis of different experimental evidence (1-6). However, no detailed data have been reported on the identity of the codons in the various species. By use of synthetic polynucleotides of known composition or trinucleotides of known sequence, the genetic code has been almost completely established in *Escherichia coli* (7-9), but similar data from other species are scarce.

The experimental evidence upon which the universality of the genetic code is firmly based does not rule out the possibility that this

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² See Discussion of this paper conducted by Dr. M. P. Stulberg: p. 193.

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code may be partially universal. Thus, one or more synonymous codons found in *E. coli* may not be present in other species, or codons not found in *E. coli* may be used by other species as a synonymous codon for a certain amino acid.

Because of the importance of this issue, the genetic code was studied in detail in several species with the aid of synthetic polynucleotides. This paper presents pertinent results from these experiments. The data indicate that the codons found in two species, chick and wheat, coincide almost perfectly with those previously reported for *E. coli*. A difference has been found in wheat germ where serine does not reveal a codon present in both *E. coli* and chick embryo.

METHODS AND PREPARATIONS

S-30 fraction from chick embryo.—Eleven-day chick embryos were homogenized for 2 minutes with 1.8 volumes of cold 0.05 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose. The homogenate was centrifuged at 0 C for 15 minutes at $15,000 \times g$, and the supernatant at $30,000 \times g$ for 30 minutes at 0 C in a Spinco model L ultracentrifuge. The supernatant (S-30 fraction) was dialyzed for 8 hours against 3 changes of the homogenizing solution, lyophilized, and stored at 20 C. Under these conditions, the lyophilized fraction is stable for at least 3 months. Before use, the dry powder was suspended in water in the proportion of 0.12 g of powder/ml. Protein was determined by the method of Lowry (10).

Chick embryo sRNA.—sRNA was prepared by the method of Rosenbaum and Brown (11).

Synthetic polynucleotides.—Homopolymers and copolymers were prepared with polynucleotide phosphorylase from *Azotobacter vinelandii* by the method of Basilio and Ochoa (12). Base composition of the copolymers was determined as described by Smith and Markham (13).

Amino acid incorporation in the chick embryo system.—The system incorporating amino acids contained the following concentrations in a total volume of 1 ml: Tris-HCl buffer, pH 7.5, 50 mM; KCl, 65 mM; $MgCl_2$, 9 mM; mercaptoethanol, 13 mM; ATP, 1 mM; GTP, 0.24 mM; creatine phosphate, 16 mM; creatine kinase, 50 μg ; ^{14}C -labeled amino acid (specific activity 10–25 $\mu c/\mu mole$), 0.016 mM; mixture of 19 ^{12}C -amino acids, 0.05 mM each; S-30 fraction preincubated for 10 minutes at 37 C, 3.2 mg of protein; poly U, 400 μg ; and other polymers, 600 μg . Other additions are noted in the tables. After incubation for 60 minutes at 37 C, the reaction was stopped by addition of 5% TCA; the precipitate was then treated as described by Zamecnik *et al.* (14), and counted in a Nuclear-Chicago gas-flow counter with an efficiency

of 39%. When C-rich polymers were used, the precipitation agent was 20% TCA. For the A-rich polymers, a mixture of 5% TCA and 0.25% sodium tungstate, adjusted to pH 2, was utilized.

Preparations from wheat germ.—Ribosomes, sRNA, and the system incorporating amino acids from wheat germ were prepared as previously described (6).

RESULTS

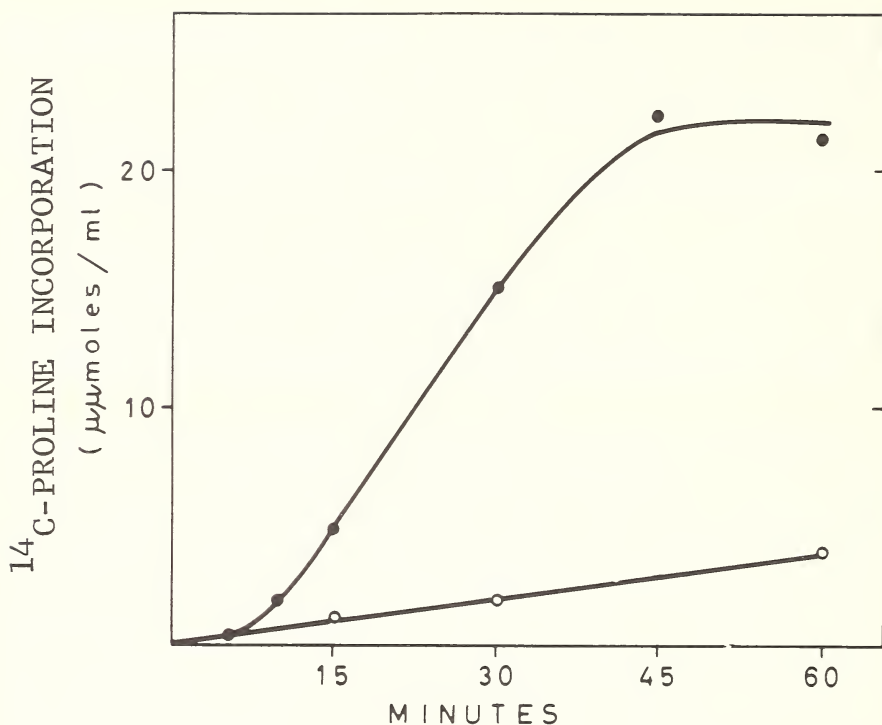
Effect of Homopolymers and General Properties of the Chick Embryo System

Investigation of the incorporation of different ^{14}C -labeled amino acids into an acid-insoluble product by a chick-embryo S-30 fraction showed that, of 17 amino acids tested individually (asparagine, glutamine, and cysteine were not available), only the incorporation of phenylalanine, lysine, and proline was markedly stimulated by poly U, poly A, and poly C, respectively. Thus, in one experiment poly U, poly A, and poly C promoted the incorporation of 788 $\mu\text{mole/ml}$ of phenylalanine, 40 $\mu\text{mole/ml}$ of lysine, and 139 $\mu\text{mole/ml}$ of proline, respectively. It appears, therefore, that UUU codes for phenylalanine, AAA for lysine, and CCC for proline. Table 1 summarizes some properties of the system incorporating amino acids in the chick embryo. Addition of poly C increased the proline incorporation almost fivefold. This incorporation was inhibited 50% by puromycin at the concentration of 10 $\mu\text{g/ml}$ and by the addition of 250 μg of chick embryo sRNA. The incorporation depended on energy and magnesium ions, and appeared to be independent of the presence of GTP, creatine kinase, and 2-mercaptoethanol. Ribonuclease completely abolished the incorporation of proline.

TABLE 1.—Properties of the poly-C-stimulated chick-embryo system

Incubation system	Proline incorporation ($\mu\text{mole/ml}$)	Percent activity
Complete*.....	180.0	100
– Poly C.....	40.8	22
– ATP and energy mix.....	10.6	6
– MgCl_2	9.4	6
– GTP.....	185.0	103
– 2-Mercaptoethanol.....	192.0	106
+ Puromycin (10 $\mu\text{g/ml}$).....	74.0	41
+ sRNA (250 μg).....	92.4	50
+ Ribonuclease (50 μg).....	24.6	13

*The complete incubation mixture contained 600 $\mu\text{g/ml}$ of poly C.



TEXT-FIGURE 1.—Effect of time on the incorporation of ^{14}C -labeled proline in the poly-C-stimulated chick-embryo system. ○—○, without polymer; ●—●, with 600 μg of poly C/ml of incubation mixture.

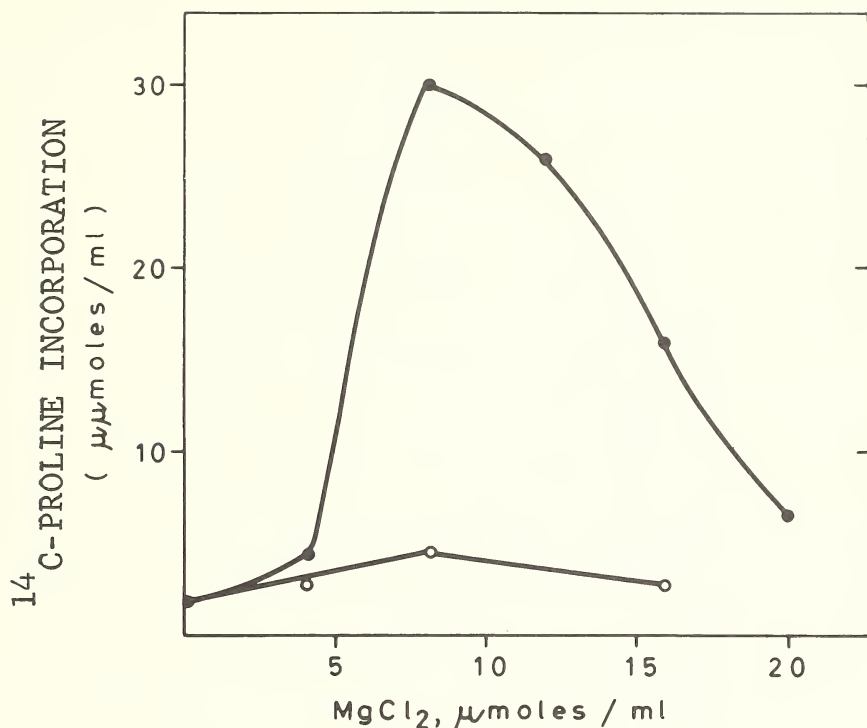
The time curve of proline incorporation in the presence of poly C is shown in text-figure 1. It presents a lag period of about 5 minutes, followed by a linear increase of up to 45 minutes.

Text-figure 2 shows the incorporation of proline at different concentrations of magnesium ions, the optimum being at 9 mM.

Effect of Synthetic Copolymers and Codon Assignment

The results of these experiments are summarized in table 2. Poly UC (3:1) and CU (2:1) stimulated the incorporation of phenylalanine, leucine, serine, and proline; poly AC (3:1) and CA (3:1) promoted the incorporation of lysine, proline, threonine, and histidine. Poly UA (2:1) directed the incorporation of phenylalanine, isoleucine, leucine, tyrosine, and lysine.

Codon assignment was made by comparing the frequency of the different triplets in the copolymers with the relative incorporation of the amino acids. On this basis, the following codons have been assigned: phenylalanine—UUU, 2U1C; leucine—2U1C, 2C1U, 2U1A;



TEXT-FIGURE 2.—Effect of magnesium ion concentration on the incorporation of ¹⁴C-labeled proline in the poly-C-stimulated chick-embryo system. ●—●, with 600 μg of poly C/ml of incubation mixture; ○—○, without poly C.

serine—2U1C, 2C1U; proline—CCC, 2C1U, 2C1A; lysine—AAA, 2A1U; threonine—2A1C, 2C1A; histidine—2C1A; isoleucine—2U1A; and tyrosine—2U1A.

Copolymers with inverse base ratios, and therefore with inverse frequency of triplets, were used to confirm the correctness of codon assignment. Thus, phenylalanine has the codon UUU; however, its incorporation as stimulated by poly CU (2:1) allows us to assign it a codon of the type 2U1C. Therefore, the incorporation of phenylalanine in the presence of poly UC (3:1) and poly CU (2:1) is directed by both the UUU and 2U1C triplets. Similarly, proline has the codon CCC, but its incorporation, as promoted by poly UC (3:1), is high enough for us to assign it a codon containing 2C and 1U. This fact has been taken into account in assigning the codons to the other amino acids.

Effects of Homopolymer and General Properties of the Wheat Germ System

The results appearing in table 3 confirm the report of Marcus and Feeley (15), with respect to the low endogenous activity of the sys-

TABLE 2.—Amino acid incorporation in chick embryo system with various polynucleotides*

Polynucleotides and experiment No.											
Amino acids	None					UC (3:1)	CU (2:1)	AC (3:1)	CA (3:1)	UA (2:1)	Codons
	1	2	3	4	5						
	Phenylalanine.....	20.4	24.0	12.0	16.5	31.6	113.6	12.8	0.0	0.0	
Leucine.....	10.0	26.0	8.6	9.3	8.2	37.2	20.4	0.3	0.0	48.8	2U1C, 2C1U, 2U1A
Serine.....	8.8	12.0	14.2	8.3	24.0	38.0	24.4	0.0	0.0	0.4	2U1C, 2C1U
Proline.....	13.2	20.4	11.2	8.4	19.2	14.4	52.4	11.0	39.6	0.0	CCG†, 2C1U, 2C1A
Lysine.....	8.4	22.3	10.0	21.6	18.4	0.0	0.7	40.0	4.8	7.2	AAU†, 2AU
Threonine.....	12.2	25.8	6.0	6.0	15.7	0.0	0.0	17.6	12.0	0.0	2A1C, 2C1A
Histidine.....	11.1	19.2	14.5	14.8	23.4	0.0	0.6	5.1	10.2	0.0	2C1A
Isoleucine.....	13.2	21.0	9.4	10.0	15.4	0.8	0.0	0.0	0.0	36.8	2U1A
Tyrosine.....	9.0	11.7	8.1	10.2	14.8	0.0	0.2	0.0	0.0	32.0	2U1A

* μ mole/ml of incubation mixture. Values without polynucleotide are subtracted from those with polynucleotide. In all, 17 amino acids were tested individually, but the ones giving negative results have been omitted from the table.

†Codons assigned by homopolymers.

TABLE 3.—Properties of the poly U-stimulated system*

Experiment No.	Incubation system	cpm of ^{14}C -phenylalanine incorporated	Percent activity
1	Complete.....	1,935	100
	— Ribosomes.....	13	1
	— Poly U.....	62	3
	— sRNA.....	498	25
	— ATP.....	693	36
	— ATP and energy mix.....	14	1
	— Mg^{++}	16	1
	— GSH.....	2,034	105
	+ Puromycin (0.5 mM).....	325	17
	+ CM (0.1 mg).....	1,714	89
	+ Ribonuclease (50 μg).....	85	4
2	Complete.....	4,040	100
	— GTP.....	1,470	36
	+ ^{12}C -amino acid mix†.....	3,850	96
	0 time.....	17	1

*The conditions of the incubation and the concentrations of the reagents were as described in "Methods and Preparations." In experiment 1, ribosomes containing 265 μg of RNA, 125 μg of poly U, and 150 μg of wheat germ sRNA were used. In experiment 2, ribosomes with 107 μg RNA, 41 μg of poly U, and 164 μg of yeast sRNA were added.

† The amino acid mix contained a final concentration of 0.05 mM of each of the natural amino acids except phenylalanine.

tem obtained from ungerminated seed. However, of 17 ^{14}C -labeled amino acids tested individually, polyuridylic acid specifically stimulated the incorporation of phenylalanine more than 100-fold. Therefore, UUU appears to be the codon for phenylalanine in this system.

Other homopolymers were inactive, although a slight incorporation of lysine and proline was repeatedly observed (not shown in table 3) with poly A and poly C, respectively. Poly I did not stimulate the incorporation of any of the different amino acids. This system has an absolute requirement for ribosomal particles, for ATP and its generating system, and for magnesium ions. Partial dependence was observed for ATP, GTP, and sRNA, whereas glutathione and the other 19 amino acids were not required. Puromycin and ribonuclease inhibited most of the phenylalanine incorporation, whereas chloramphenicol had a negligible effect at the concentration used.

Effect of Synthetic Copolymers and Codon Assignment

The specific incorporation of amino acids in the presence of U-rich copolymers is summarized in table 4. Poly UC (3:1) stimulated the incorporation of phenylalanine, leucine, proline, and serine; poly UG (5:1) directed the incorporation of phenylalanine, leucine, valine, glycine, and tryptophan. The incorporation of valine with this last copolymer was unexpectedly high. Poly UA (5:1) promoted the incorporation of phenylalanine, leucine, isoleucine, tyrosine, and lysine; and poly UAG (6:1:1) stimulated the incorporation of phenylala-

nine, leucine, isoleucine, glutamic acid, aspartic acid, tyrosine, and methionine.

The codon assignment, shown in the last column of table 4, was deduced in the same way as in the chick embryo system.

DISCUSSION

The two systems described incorporating amino acids possess general characteristics similar to those described for preparations obtained from other sources. Both chick embryo and wheat germ systems respond to synthetic polynucleotides, and both have an absolute requirement for energy and for magnesium ions. Puromycin and ribonuclease inhibit the polynucleotide-stimulated incorporation of the amino acids, and sulfhydryl compounds are not essential for activity.

There are, however, a few differences between the two systems that deserve notice. Whereas GTP appears to be required in wheat, it has no effect in chick embryo. Since the latter system is a crude S-30 fraction, contamination with GTP is highly probable; hence, no effect would be observed by the extra addition of GTP. Wheat germ is dependent on sRNA, whereas chick embryo is inhibited by this component. Inhibition of the amino acid incorporation by addition of sRNA has been described by Maxwell (16) and Decken and Campbell (17) in similar systems obtained from rat liver. An explanation of the inverted effect of sRNA may reside in the fact that the S-30 fraction contains enough sRNA for protein synthesis and that the extra addition of sRNA would decrease, by binding, the magnesium ion concentration. It should be recalled that the chick embryo shows a rather sharp optimal concentration for magnesium ions.

The codon assignment in both systems coincides almost perfectly with that previously reported for *E. coli*. However, a few discrepancies should be noted. In wheat germ, valine showed an incorporation, directed by poly UG (5:1), high enough to justify the assignment of three codons (one permutation of the type 2G1U and two of the type 2U1G). Since leucine has been assigned a 2U1G codon, cysteine could not have a similar permutation, as found in *E. coli*. Cysteine was not tested, and the significance of these results is therefore uncertain.

In chick embryo, lysine shows two codons, AAA and 2A1U. The latter codon has also been assigned to lysine in wheat germ and *E. coli* as a result of experiments with poly UA (18). However, in *E. coli*, experiments with trinucleotides of known sequence containing permutations of the type 2A1U have been negative (8). It is possible that a low affinity of these trinucleotides for the ribosomes of *E. coli* may explain the failure to bind lysyl sRNA.

TABLE 4.—Amino acid incorporation in wheat germ system with various polynucleotides*

Amino acid	Polynucleotides and experiment No.							
	None							
	1	2	3	4	UC (3:1)	UG (5:1)	UA (5:1)	UAG (6:1:1)
					1	2	3	4
Phenylalanine.....	10.0	16.0	25.0	16.0	432.0	315.0	315.0	163.5
Leucine.....	18.4	18.3	26.5	28.3	235.6	90.6	77.5	85.0
Proline.....	8.0	3.2	6.6	—	88.5	0.0	0.6	—
Serine.....	7.3	2.7	11.4	—	91.9	0.3	0.0	—
Glycine.....	6.8	4.1	7.8	—	0.4	9.9	0.4	—
Isoleucine.....	9.6	3.8	12.0	14.0	0.0	0.0	55.4	30.6
Glutamic acid.....	9.3	3.2	4.2	6.4	0.0	0.0	2.1	11.0
Aspartic acid.....	4.4	2.1	3.8	19.1	1.2	0.2	6.3	15.2
Lysine.....	7.4	2.9	32.0	—	0.0	0.0	19.0	—
Tyrosine.....	8.3	4.2	10.5	12.6	0.0	0.0	49.5	39.5
Methionine.....	7.4	2.9	9.2	1.2	0.0	0.1	0.0	9.3
Tryptophan.....	5.5	7.9	4.4	—	1.7	22.1	0.0	—
Valine.....	6.7	4.7	9.2	—	0.0	182.3	0.0	—

* μ moles/mg of ribosomal RNA. Values without polynucleotide are subtracted from those with polynucleotide. In all, 17 amino acids were tested individually [except for poly UAG (6:1:1)], but the ones giving negative results have been omitted from the table.

In chick embryo, serine has been assigned two codons, 2U1C and 2C1U. The same codons were found in *E. coli*. In wheat germ, however, only the codon 2C1U could be assigned. The 2U1C codon, which is easier to detect with poly UC (3:1) than is the 2C1U codon, appears to be absent. The significance of this finding is obscure and may be true only in the wheat germ and not in the adult plant. Possibly, in the wheat germ, the sRNA corresponding to the absent codon is not present. Since the availability of sRNA has been postulated as a control mechanism in protein synthesis (19), this possibility should be investigated further with respect to the development of the wheat plant.

The rest of the codons assigned in both chick embryo and wheat germ are identical to those reported for *E. coli*, providing additional support to the universality of the genetic code.

The fact that the genetic code appears to be universal gives rise to the question: Why is this code the same in all living organisms despite the fact that most of the components involved in protein synthesis have changed throughout evolution? It is usually argued (20) that once a complete code has arisen it would be very difficult to change, since any alteration in the meaning of a codon would change the primary structure of almost every protein in the organism and would, in all probability, be lethal. A change in the meaning of codon would occur only if an aminoacyl-sRNA synthetase changes its specificity for one amino acid to another amino acid. This is not a change in the genetic code, but a change in the genetic information of the structural gene of this particular aminoacyl-sRNA synthetase. The same would be true if the recognition site of a certain sRNA for the corresponding aminoacyl-sRNA synthetase changes in such a way that can be recognized by a different aminoacyl-sRNA synthetase. This last possibility assumes that the anticodon is not part of the recognition site. A change in the code should be understood as a proper and specific change in every component of the informational system without altering the meaning of the information. For instance, according to this definition, if the codon UUU would change its meaning to lysine, and the codon AAA to phenylalanine, then every TTT triplet in DNA (complementary to the AAA codon of lysine) should be replaced by AAA triplets, and every AAA triplet in DNA (complementary to the UUU codon of phenylalanine) should be replaced by TTT triplets. It would also be necessary to have specific changes in the structural genes of the phenylalanyl and lysyl-sRNA synthetases that would result in a reciprocal change of their specificity. The fact that these multiple, simultaneous, and specific changes are absolutely improbable provides a satisfactory explanation for the lack of diversification of the genetic code throughout evolution.

The invariability of the code has also been explained by assuming that its translation is not genetically determined and that a stereochemical interaction takes place between the amino acid and the anticodon present in the corresponding sRNA (1). Such interactions have not been reported. Pelc and Welton (21), using molecular models, recently found a structural relationship between coding triplets and amino acids. Related experiments have also been published by Woese *et al.* (22). The relationship between codon and amino acids has been taken as a factor involved in the determination of the codons presently observed at some primitive stages of evolution. Since it is difficult to assess these data, it is necessary to wait for more direct experimental evidence of this nature.

RESUMEN

Se prepararon sistemas incorporadores de aminoácidos de embriones de pollo y germen de trigo. Ambos sistemas responden a polinucleótidos sintéticos y dependen en forma absoluta de energía y de iones magnesio. La incorporación de aminoácidos estimulada por polinucleótidos es inhibida por puromicina y por ribonucleasa. Los grupos sulfhidrilos no son esenciales. El sistema obtenido de germen de trigo depende de la presencia de GTP y sRNA. En cambio el sistema de pollo no requiere GTP y es inhibido por la adición de sRNA.

Usando polinucleótidos sintéticos de composición conocida como mensajeros artificiales se pudo, tanto en trigo como en pollo, obtener los condones para los diferentes aminoácidos. Los codones encontrados son casi iguales a los de *Escherichia Coli* haciendo excepción sólo la serina que en el sistema de trigo no muestra el codon 2U1C que está presente en *E. coli* y en el embrión de pollo. Se discute el significado de este hallazgo en relación a un posible mecanismo de control de la síntesis de proteínas.

Se da una posible explicación de la universalidad del código genético sobre la base de la improbabilidad casi absoluta de que ocurran los cambios que serían necesarios para cambiar el código.

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DISCUSSION ¹

Discussor, DR. M. P. STULBERG, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

I WOULD like to compliment Drs. Basilio and Allende on developing such an excellent system from wheat germ. The very low endogenous incorporation and high stimulation by synthetic messenger have allowed Dr. Basilio to accumulate rather complete coding information in a relatively short time. Certainly this system should be amenable to the examination of mechanisms of control and differentiation in wheat tissue. Dr. Allende's experiments have shown increased homogeneity in valine and methionine tRNA upon germination of the wheat embryo, and are in agreement with those data concerning sporulation of bacteria, where greater heterogeneity of tRNA seems to exist in the dormant state (spores). Certainly the differences in tRNA noted between the seed and germinating tissue should be reexamined with fractionating procedures of greater resolving power. This conclusion also applies to Dr. Basilio's wheat-germ system where the absence of a 2U1C codon for serine should encourage him to search for an additional serine tRNA in the germinating tissue.

If I might digress for a moment, I would like to speculate on another possible focal point for regulation of protein synthesis involving the -CCA terminus of the tRNA molecule. The *in vivo* turnover of this terminus seems to vary, depending on the tissue examined and the state of growth of the tissue. For example, Drs. Wicks, Greenman, and Kenney have examined fetal rat liver and observed that the -CCA terminus of tRNA is quite stable *in vivo*. This is in striking contrast to the situation in adult liver where the terminus turns over quite rapidly. These types of differences could be a reflection of regulatory action. When any part of the terminus is missing, the tRNA cannot accept amino acids and thus could have a significant effect on protein synthesis. Furthermore, we and other investigators have shown that once the -CCA terminus is altered the remainder of the tRNA mole-

¹ Of articles by Jorge E. Allende; and Carlos Basilio.

cule becomes an effective inhibitor of aminoacyl-tRNA formation. In connection with these studies, we have observed that elimination or periodate oxidation of the terminus causes a significant change in the optical rotary dispersion (ORD) spectra of phenylalanine tRNA, suggesting a large change in the conformation of the molecule. This type of ORD change has also been observed by Zamcenik's group upon aminocylation of tRNA. Thus one is led to believe that the -CCA terminus is not only important as the acceptance site of an amino acid but also is crucially involved in the conformation of the tRNA molecule. One can thus speculate on the possibility that a conformationally changed tRNA could effect protein synthesis also at the translational level.

Audience discussion was initiated by G. D. Novelli who commented on the changes in chromatographic patterns of tRNA as described by Allende. He mentioned the work of Sueoka's group, concerning changes in leucine tRNA after phage infection of *E. coli*, as being similar in nature to those of Allende. Using the reversed phase chromatography method developed at Oak Ridge, Novelli's group examined the phenomena observed by Sueoka in more detail. This procedure resolves leucine tRNA into five species. They repeated Sueoka's experiments and confirmed their observation, but in addition noted sharp increases in peaks 4 and 5 after prolonged infection of *E. coli* with T2 phage. An experiment designed to examine leucine tRNA patterns during the full time course of infection revealed that the increases observed in peaks 4 and 5 occur just prior to lysis of the cells. Thus these changes seem to be correlated with a late virus function or possibly final maturation of the phage. Novelli also mentioned the work of Barnett who has observed two specific phenylalanyl-RNA synthetases and two specific phenylalanine tRNAs in *Neurospora* that do not cross-react. Barnett has further shown that one of the synthetases is of mitochondrial origin. Novelli's group has applied the *Neurospora* tRNA to reversed phase chromatography and found that one of the tRNAs is also of mitochondrial origin. Thus *Neurospora* seems to have distinct sets of tRNAs and synthetases in mitochondria and cytoplasm, respectively. Finally, Novelli mentioned that Barnett had also demonstrated that the code assignments in *Neurospora* were identical to those of *E. coli*.

Dr. Marroquin inquired about the almost absolute requirement for GTP in the synthesis of polyphenylalanine as demonstrated by Dr. Allende in his wheat-embryo transfer system. He raised the question whether the GTP might not be acting by completing an otherwise incomplete tRNA and thus causing the stimulation noted. Dr. Allende

said that they had not tested the requirement for GTP in the more purified transfer system beyond its stimulating properties and did not know whether it was incorporated into tRNA. However, he had examined this possibility when working with an *E. coli* system in Lipmann's laboratory and found no incorporation of GTP into nucleic acids.

Dr. Ondarza injected a word of caution concerning the postulation that "lack of messenger" might be the reason for the inactivity of the endogenous incorporation system from ungerminated wheat embryos. He related the situation with α - and β -amylases where they show large increases in activity upon germination of the seeds. This increase is at least partially due to the activation of bound, latent enzymes that can be activated by proteolytic action.

Dr. Reynosa questioned whether the slight decrease of amino acid incorporation noted upon the addition of GTP in one experiment might be due to the complexing of Mg^{++} . The consensus was that this inhibitory effect was due to a lowering of the Mg^{++} level, below its critical concentration, by binding to excess GTP. Stulberg reemphasized that the 50% inhibition of proline incorporation noted in the chick embryo system upon addition of tRNA might be due to "scavenging" of Mg^{++} . This possibility has not been directly tested.

WEDNESDAY MORNING

Chairman: Barbarín Arreguín

Enzyme Induction by Bacteriophage^{1,2,3}

E. VOLKIN, *Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831*

SUMMARY

The insertion of bacteriophage DNA into the host cell results in a striking and almost immediate transition in the synthesis of all macromolecular species. Bacterial DNA synthesis stops and, in fact, this DNA becomes progressively degraded. The degradation products (as well as exogenous material and some RNA) become the source for the formation of viral DNA. The conversion is brought about by the action of a large group of newly induced enzymes whose combination of specificities is ideally suited for the purpose. It is clear that the messages for directing the synthesis of these early enzymes must be formed almost instantaneously after phage infection. Actually, it had been determined long before the discovery of the induced enzymes that bacteriophage infection results in a prompt block in the net synthesis of RNA. Specific messenger RNA (mRNA) is synthesized at a vigorous rate and

this RNA undergoes a constant turnover so that there is essentially no accumulation of it under normal conditions. The proteins produced subsequent to phage infection, then, appear to be exclusively those associated with the production of viral progeny: induced enzymes concerned with the production of viral DNA, lysozyme, and those proteins that constitute the various structural components of the virus particle. A considerable effort by many laboratories is focused toward the problem of the temporal sequence of formation of specific mRNAs and specific proteins. It seems that a control mechanism permits the synthesis of some mRNAs to appear early, then cease, while other mRNAs are made continuously and others only relatively late in the viral growth cycle. Correspondingly, the proteins specified by these RNAs are sequentially produced. —Nat Cancer Inst Monogr 27: 199–210, 1967.

BACTERIOPHAGE INFECTION represents an ideal *in vivo* system for demonstrating the role of DNA as the primary agent in the control of specific protein synthesis. The processes of phage infection, like

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. E. L. Tatum, p. 243.

³ Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

bacterial transformation, are determined solely by the entering viral DNA; but unlike transformation systems, the entire population of host bacteria is affected by the entering DNA. The alteration in metabolic functions is an almost immediate one and encompasses the complete spectrum of macromolecular events: a cessation of host DNA synthesis and vigorous replication of phage DNA; an inhibition of further RNA synthesis except for the production and turnover of viral messenger RNA (mRNA); and the synthesis of a large number of enzymes specifically designed to produce viral progeny in the most efficient way. I should like to describe some basic aspects of this example of an immense switch in metabolic regulation, restricting this discussion to the case of infection with the T phages.

VIRAL DNA REPLICATION

Hershey and Chase (1) demonstrated clearly that bacteriophage DNA and not the protein enters the host cell. Thus the viral DNA molecule is available for the transcription of its genetic functions. The question that is immediately apparent is how this quantitatively minor fraction of DNA competes so successfully with the host DNA. Actually there is no such competition, because the bacterial DNA very quickly becomes fragmented to low-molecular weight nucleotides, which are, in fact, used as precursors for the synthesis of progeny phage DNA (2, 3). Is the enzyme (or enzymes) that is responsible for host DNA breakdown due to an activation of preformed bacterial deoxyribonuclease or to the formation of a new enzyme induced by the introduction of the viral genome? Stone and Burton (4) have shown that a new deoxyribonuclease (DNase) is formed after infection with T2, T5, or T6 and that this activity cannot have resided in an inhibited form in the uninfected bacteria. It has not been resolved, however, whether or not this DNase activity is responsible for host cell DNA degradation. Another question that can be raised is how the viral DNA remains immune to enzymatic hydrolysis. Here again, the answer is not clear, but it is known that the DNAs from T-even phages (which contain glucosylated forms of the base, hydroxymethylcytosine) are relatively resistant to various nucleases (5, 6). Y. T. Lanni and D. J. McCorquodale (7, 8) have shown that host-cell DNA breakdown is induced by only a fragment of the T5 DNA (about 10% of the viral DNA) that initially enters the host bacterium. Thus one of the earliest manifestations created by introducing the phage genome is the destruction of the genes of the host, but it remains to be proved whether this function results from an activation of preformed enzyme or from a newly induced enzyme.

The replication of the DNA of the T-even phages requires that the host produce a molecule of nucleotide composition widely different from that synthesized before infection, that a new base be synthesized (hydroxymethylcytosine in place of cytosine), and that this base be glucosylated. This is accomplished by the *de novo* formation of a collection of induced enzymes whose combination of activities is ideally suited for the above purposes. A list of these induced enzymes responsible for T-even phage DNA synthesis follows: deoxyguanylate kinase (9), deoxyuridylate kinase (10), deoxycytidylate pyrophosphatase (9), deoxycytidylate hydroxymethylase (9, 11), deoxycytidylate deaminase (12), thymidylate synthetase (13, 14), various glucosyl transferase (9, 15), dihydrofolate reductase (16), and polymerase(s) (17).

It should be noted that the induced enzymes involved in viral DNA synthesis are called "early" enzymes because their synthesis starts within a minute or two after infection and then ceases before the midpoint of the latent period. The characteristic rapid synthesis of "early" enzymes during just the first 10 minutes after infection and the virtual cessation of synthesis thereafter make it appear that some mechanism involves sequential control of either *a*) the transcription of the genome, or *b*) the reading of the various RNAs if these are all produced early. Many of the amber mutants of T4, when infecting *Escherichia coli* B, fail to synthesize one or more of these early enzymes and, in some cases, create an overproduction of certain enzymes (18).

It is interesting that a new DNA polymerase is induced by infection, since the *in vitro* requirements of the bacterial enzyme and of the phage-induced enzyme are essentially identical. A difference that exists between the two is that the phage-induced enzyme functions much better with the denatured, or single-stranded, DNA template. Perhaps this is related to the increasing number of observations (19–21) that the *in vivo* replicative form of viral DNA is structurally modified compared with the form of the DNA that exists in the intact phage particle. Such structurally modified replicative forms (21) have been invoked to account for the circularly permuted molecules of DNA found (22) in a normal population of T-even phage.

Changes in structure of viral parental DNA likewise are being studied to elucidate the mechanism of genetic recombination—a process which takes place with high efficiency in these systems and which, apparently, does not require DNA replication. I should like to discuss briefly some possible enzymological aspects of the phenomenon of genetic recombination. First, to build a framework for consideration of the data to be presented, I shall state two conclusions that appear to be well founded: *a*) Genetic recombination in bacteriophage involves some process of breakage of parental genomes and rejoining of the fragments (23); and *b*) parental phage DNA is found in the progeny

DNA as relatively small fragments—not as conserved or semiconserved DNA strands (20).

Dr. Mead (24), in our laboratory, has isolated an enzyme fraction from T2-infected bacteria whose properties could conceivably make it a candidate as an enzyme to carry out rejoining of DNA fragments. The enzyme attaches oligodeoxyribonucleotides to double-stranded DNA. The requirements of the system are shown in table 1. While the enzyme has not been shown to be one necessarily induced by T2 infection, it is present in much higher activity after infection, and the enzyme isolated from infected cells shows some degree of specificity toward utilizing T2 DNA as the acceptor molecule (table 2). Unlike polymerase, the enzyme will not utilize deoxyribonucleoside triphosphates, works poorly with denatured DNA as acceptor, and can be physically separated from polymerase activity. The enzyme has now been completely freed of exonuclease activity but an endonuclease activity is retained with the enzyme through various modes of purifi-

TABLE 1.—Reaction requirements

Reaction mixture	Total counts incorporated	μ moles oligonucleotide phosphorus incorporated
Complete.....	1,950	790
— Sonicated DNA.....	200	1
— Mg^{++}	270	32
— ATP.....	197	1
— ATP generating system.....	1,440	550

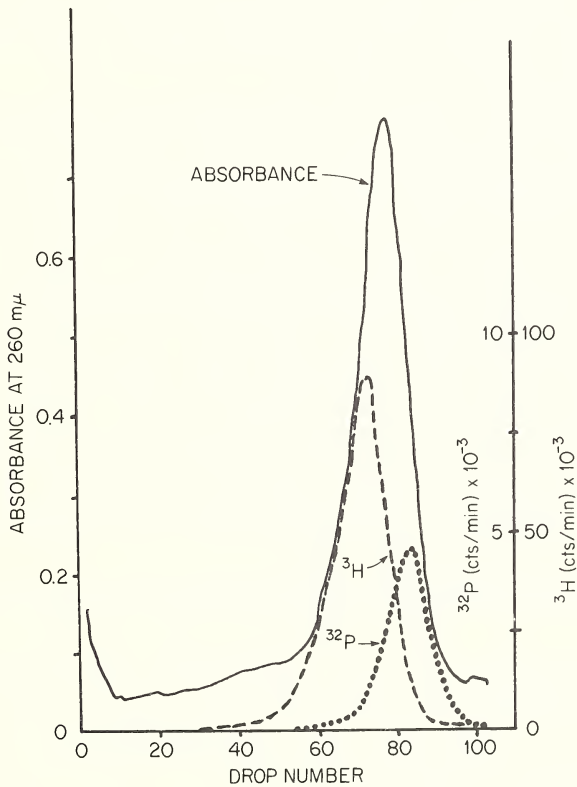
*The complete reaction mixture (0.5 ml) contained 25 μ moles of Tris-Cl buffer, pH 9; 5 μ moles of $MgCl_2$; 0.5 μ moles of ATP; μg of sonicated T2 DNA; 0.05–0.2 μc of ^{32}P -labeled oligodeoxyribonucleotides; and 100 μg of enzyme protein. The incubation was carried out at 37 C, and aliquots were removed every 10 minutes and processed. The initial rate of the reaction was determined and is expressed as μ moles of oligonucleotide phosphorus incorporated per hour.

TABLE 2.—Acceptor efficiency*

Acceptor polynucleotide (40 μg /0.5 ml reaction)	Total counts incorporated	μ moles oligonucleotide phosphorus incorporated	Relative acceptor
Sonicated			
T2 DNA.....	3,090	1,545	100
<i>E. coli</i> DNA.....	323	163	11
Native			
T2 DNA.....	2,660	1,330	86
<i>E. coli</i> DNA.....	400	200	13

*Native DNA preparations were isolated by the method of Astrachan, Volkin, and Jones (25). Sonication was carried out with a Raytheon 200 watt, 10 Kc magnetostriuctive oscillator at maximum power for 3 minutes at 0 C.

cation. The endonuclease activity is of particular interest because the product of the hydrolysis reaction, with native T2 DNA or sonicated T2 DNA as substrate, is of relatively large size (about 1–5 million according to present, preliminary estimates), and the product seems to be fairly homogeneous with respect to its sedimentation in a sucrose gradient. It may be of special significance that the product of the oligodeoxyribonucleotide transferase reaction is of this same size range (text-fig. 1). It remains to be determined whether the endonuclease activity of the acceptor DNA is an integral initial requirement before attachment of oligodeoxyribonucleotide can occur, or whether the enzyme is simply a contaminant that splits the product of the attachment reaction into somewhat smaller molecules.



TEXT-FIGURE 1.—Sucrose density gradient centrifugation of the product. The product was isolated from a reaction by chromatography on a MAK column after 60 minutes of incubation and analyzed by sucrose density gradient centrifugation on a 5–20% sucrose gradient in SSC. The sample was centrifuged for 4 hours at 4 C at 38,000 rpm in an SW39 rotor. *Escherichia coli* DNA labeled with ³H-thymidine was added as a marker.

MESSENGER RNA

One of the earliest biochemical observations (26) on virulent phage-infected bacteria was that total RNA synthesis immediately ceases upon infection. Our work of 1956 (27) confirmed this, but also established that a minor RNA component was continually being synthesized and degraded during the course of infection (28). This RNA fraction incorporated isotope into its constituent nucleotides in a manner that made it appear to have a primary structure similar to the phage DNA. Subsequently, this discovery held a key role in the postulate that such RNA (now called messenger RNA) is transcribed from DNA and serves as the direct coding agent for the synthesis of specific proteins. I do not intend to review the multitude of evidence that has accumulated which strongly substantiates the role of mRNA and which more completely describes its properties and specificity. Instead, I prefer to consider some studies on phage mRNA relevant to the problem of the induction of specific enzymes.

The synthesis of the bulk of T2-specific mRNA is dependent on the synthesis of new protein. One of our early findings (29) revealed that if the protein synthesis inhibitor, chloramphenicol, is added a few minutes before infection, bacterial-like RNA is synthesized, whereas if the inhibitor is added a few minutes after infection, phage mRNA is produced (table 3). This result indicates that a specific protein is made immediately after infection which is responsible for the formation of the viral-specific RNA. But how is the mRNA made for this particular protein? We conjecture that a host enzyme (host RNA polymerase?) catalyzes the synthesis of mRNA that codes for the synthesis of that protein (phage RNA polymerase?) that catalyzes the synthesis of most of the phage-specific mRNAs. This implies that the first viral gene transcribed that involves induction of a new protein must direct the synthesis of phage-specific RNA polymerase.

TABLE 3.—Effects of chloramphenicol on relative specific activities of RNA mononucleotides*

Mononucleotide	Specific activity (counts/sec/ μ g of P) of mononucleotides under indicated conditions		
	No. CP	CP added 3 minutes after infection	CP added 3 minutes before infection
Cytidylic acid	1.2	1.1	1.0
Adenylic acid	1.8	2.1	1.0
Uridylic acid	1.9	2.1	1.2
Guanylic acid	1.0	1.0	1.0

*45 μ g/ml chloramphenicol (CP) was used.³²PO₄ added at 6 minutes after T2; samples removed 8 minutes later. RNA mononucleotides, released by alkaline hydrolysis of RNA, were separated by ion-exchange procedures. Recorded are specific activities relative to that of guanylic acid.

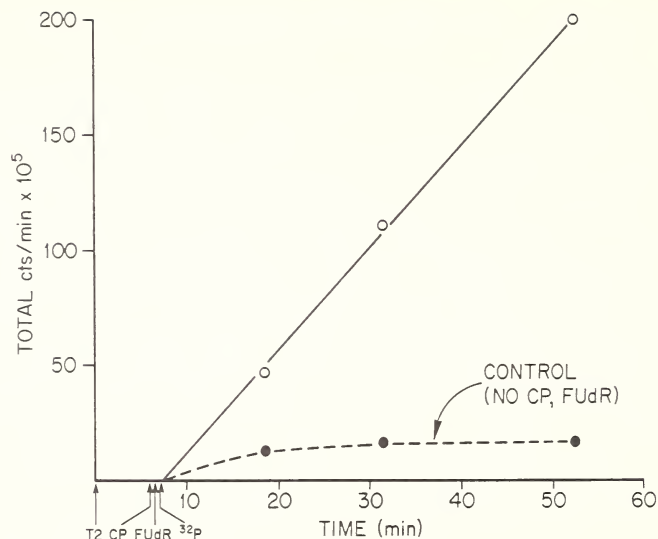
The experiments with chloramphenicol revealed another interesting facet of the synthesis of mRNA, *i.e.*, that the prevention of protein synthesis blocks the turnover of mRNA. We are presently attempting to exploit this phenomenon for the purpose of accumulating and preparing relatively large quantities of T2-specific mRNAs. The addition of chloramphenicol some 6 minutes after infection with T2 allows the synthesis, without noticeable degradation, of some T2 mRNA, but also allows the continued and vigorous synthesis of T2 DNA. By inserting an inhibitor of DNA synthesis (5-fluorodeoxyuridine) simultaneously with the addition of chloramphenicol, we can eliminate this source of massive competition for nucleic acid precursors and accumulate large amounts of what appears to be T2-specific messenger RNA. Text-figure 2 shows the continual incorporation of ^{32}P into RNA under these conditions, and the results shown in table 4 indicate that this RNA has a gross nucleotide composition similar to the T2 DNA. It is of interest that the extent of isotope incorporated into RNA in these experiments is the same order of magnitude as that calculated from a T2-infected, uninhibited culture, had there been no turnover of the mRNA. Hybridization experiments conducted in collaboration with Drs. S. K. Niyogi and W. S. Riggsby of our laboratory confirm that much, if not all, of the accumulated, labeled RNA anneals specifically to denatured T2 DNA.

TABLE 4.—Ratios of the specific activities in the RNA mononucleotides*

Mononucleotide	Minutes after infection		
	18.5	31.5	52.5
Cytidylic.....	0.97	0.97	0.95
Adenylic.....	1.86	1.77	1.70
Uridylic.....	1.82	1.71	1.72
Guanylic.....	1.0	1.0	1.0

*Data taken from the text-figure 2 experiment in which chloramphenicol and 5-fluorodeoxyuridine had been added. Numbers are relative to guanylic acid.

Specific hybridization techniques involving competition for the denatured T2 DNA template by phage mRNAs synthesized at different times after infection demonstrate that transcription is an ordered process (30, 31). However, in addition to a sequential transcription of messenger RNAs, the evidence (31) indicates that some temporal control occurs with respect to the utilization of the RNA. Thus, by applying hybridization techniques to T2 mRNAs formed in the first 6.5 minutes and 15 to 19 minutes after infection, Hall *et al.* (31) found that indeed the late RNA contained a large proportion of molecules not formed at the early time. However, so-called early mRNA was



TEXT-FIGURE 2.—RNA synthesis in the presence of chloramphenicol and 5-fluorodeoxyuridine. Five minutes before infection, 20 $\mu\text{g}/\text{ml}$ each of uridine and cytidine were added to the culture to compete with any 5-fluorouracil that may be formed and be incorporated into RNA. The following quantities were added at the times indicated: 35 $\mu\text{g}/\text{ml}$ chloramphenicol, 10 $\mu\text{g}/\text{ml}$ 5-fluorodeoxyuridine, 1.5 $\mu\text{C}/\text{ml}$ $^{32}\text{PO}_4$. The analysis for RNA mononucleotides was carried out as described in (27).

found in abundant quantity at the later time interval. Since early enzyme synthesis is known (18) to cease before 15 minutes post infection, it would appear that the control of this kind of protein synthesis resides not at the gene transcription level but at the level of translation of mRNA. Shalitin and Thomas (32) confirmed these results and, in addition, found that early mRNA resulting from T4 infection hybridized with regions on the T4 DNA molecule relatively richer than average in AT base pairs. This same result was obtained with early λ -phage messenger and that half of λ -DNA richer in AT base pairs (33). Khesin and Shemyakin (34) and Geiduschek *et al.* (35) demonstrated that essentially all of the T-even early, but none of the late, mRNA is synthesized *in vitro* by *E. coli* RNA polymerase. Bautz and co-workers (36, 37) used various deletion mutants of phage T4 to refine the DNA-RNA hybridization experiments to the point where they are now able to isolate mRNAs transcribed by specific, small segments of the T4 genome. One of the unusual findings they report (37) is that the mRNA associated with the late protein, lysozyme, is made early, but apparently not utilized at this time. The suggestion has been made that the early lysozyme mRNA, for some reason, fails

to associate with ribosomes. The rate of transcription for this RNA then declines but increases again during that time when lysozyme is synthesized.

PROTEIN SYNTHESIS

The general picture of protein synthesis after infection with virulent phage can be described as an immediate cessation in the production of host protein and a concomitant synthesis of phage-induced protein. The phage-induced protein is generally divided into "early" and "late" classes, where the former has been found to be those enzymes associated with the replication of phage DNA and the latter to be those associated with the synthesis of the various structural phage-protein elements, as well as the enzyme, lysozyme. It should be noted, however, that at least one phage protein, the "internal protein" found inside T-even phage particles, is synthesized at a faster rate in the early period post infection (38). On the other hand, though lysozyme is the only enzyme activity well documented as a late enzyme, the immunologic approach by Thomas and Suskind (39) and one that we are currently carrying out indicate that a considerable amount of T2- and T4-induced protein over and above the phage structural protein is synthesized at a late postinfection time period.

If one were to anticipate some relatively straightforward control mechanism of protein synthesis in phage-infected bacteria, in addition to the control exerted by transcribing the phage genome rather than the bacterial genome, it would likely entail a change in one or more transfer RNAs (tRNA) and/or a change in the corresponding tRNA synthetases. By this mechanism, regulation of protein synthesis would be under the control of a new translating system, one essentially foreign to the host. W. E. Barnett and I, employing antisera produced by *E. coli* and T2-infected *E. coli* soluble proteins, have made a cursory survey for such changes with little success thus far. We have found that there are wide degrees of inactivation of 16 aminoacyl synthetases by the two antisera. This variation could be because of differences in immunogenicity of these proteins or differences in the effect of the antibodies on the active enzyme sites among these enzymes. We could not detect, however, any immunologic changes in the amount or specificity of individual synthetases resulting from T2 infection. Similarly, the gross tRNA fractions from infected and uninfected cells behaved similarly toward the synthetase preparations from infected and uninfected cultures.

Two recent findings from other laboratories do denote some promise that this mechanism of control of phage-specific protein synthesis takes place. The Sueokas reported (40) that shortly after T-even

phage infection a new, or modified, leucine tRNA appears. It will be of special interest to determine whether any new coding properties can be assigned to this RNA or whether a new synthetase activity arises to match the RNA. Of particular interest is the report by Neidhardt and Earhart (41) that T4 infection induces the formation of a new valyl tRNA synthetase. Infection of an *E. coli* mutant with a temperature-sensitive synthetase results in the appearance of a temperature-resistant enzyme, while from T4-infected wild-type *E. coli* the authors could chromatographically separate a new valyl synthetase activity. The new enzyme activity can be classified as an "early" enzyme, and its synthesis is blocked by protein synthesis inhibitors.

RESUMEN

La inserción de ADN de bacteriófago en una célula huésped produce una transición notable y casi inmediata en la síntesis de todas las especies macromoleculares. La síntesis de ADN bacteriano se detiene y, de hecho, este ADN se degrada progresivamente. Los productos de degradación (así como el material exógeno y algo de ARN) se convierten en la fuente de productos para la formación del ADN viral. La conversión se lleva a cabo por la acción de un gran grupo de enzimas recién inducidas, cuya combinación de especificidades está idealmente adaptada para este fin. Es claro que los mensajes para dirigir la síntesis de estas enzimas iniciales deben formarse casi instantáneamente después de la infección del fago. En rigor, se había descubierto mucho antes de reconocimiento de que la infección por el bacteriófago causa la aparición de enzimas inducidas, que sobreviene un rápido bloqueo de la síntesis neta de ARN. El ARN mensajero específico (ARN_m) es sintetizado a gran velocidad y este ARN sufre un recambio constante de manera que prácticamente no se acumula en condiciones normales. Después, las proteínas producidas en seguida de la infección por el fago, parecen ser exclusivamente aquellas que están asociadas con la producción de la progenie viral, o sea enzimas inducidas relacionadas con la producción de ADN viral, lisozima y todas las proteínas que constituyen los diversos componentes estructurales de la partícula viral. En muchos laboratorios se están haciendo considerables esfuerzos para atacar el problema de la secuencia temporal de la formación de los ARN_m específicos y de las proteínas específicas. Es posible que un mecanismo de control permita la síntesis de algunos ARN_m que aparezca inicialmente y después se detenga, mientras que otros ARN_m se producen continuamente y, por fin, otros más sólo aparecen en las fases tardías del ciclo del crecimiento del virus. Enseguida, las proteínas específicas por estos ARN_m son producidas en el orden correspondiente.

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Regulation of Enzymes Induced by Animal Viruses^{1,2,3}

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SUMMARY

Deoxyviruses induce several enzymes concerned with DNA synthesis. As with bacteriophage-induced enzymes, their synthesis is regulated. Attention is focused mainly on two induced enzymes—thymidine kinase and a deoxyribonuclease. Although the synthesis of these enzymes appears to terminate almost simultaneously, striking differences in the mode of this regulation can be demonstrated. The synthesis of thymidine kinase and two other enzymes does not require DNA synthesis,

although termination of kinase synthesis does depend upon the formation of viral progeny DNA. In contrast, deoxyribonuclease synthesis is expressed only by progeny DNA. In addition to regulation of synthesis, the activity of induced thymidine kinase appears to be controlled by cell factors that change the configuration of the enzyme. Recent studies in the change in state of the deoxyribonuclease from soluble to particulate are described.—*Nat Cancer Inst Monogr* 27: 211–219, 1967.

ENZYMES ARE induced by poxvirus infection of mammalian cells. Synthesis of these enzymes is regulated. The activities of thymidine kinase and acid deoxyribonuclease (DNase) are also subject to regulation. Associated with the loss or regain of enzyme activity of thymidine kinase are configurational changes caused by removal or addition of substrates. The increase in the postinfection activity of soluble acid DNase is eventually arrested. This is correlated with an increase in the enzyme activity that can be liberated from a bound state.

Late poxvirus functions (the repression of early enzyme synthesis and the induction of the enzyme acid DNase) are dependent on synthesis of viral DNA. In this respect, the regulation of early enzyme

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² See Discussion of this paper conducted by Dr. E. L. Tatum, p. 243.

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synthesis in poxvirus-infected cells is similar to that in T2-infected *Escherichia coli*. A brief discussion of these phenomena in relation to virus replication is presented.

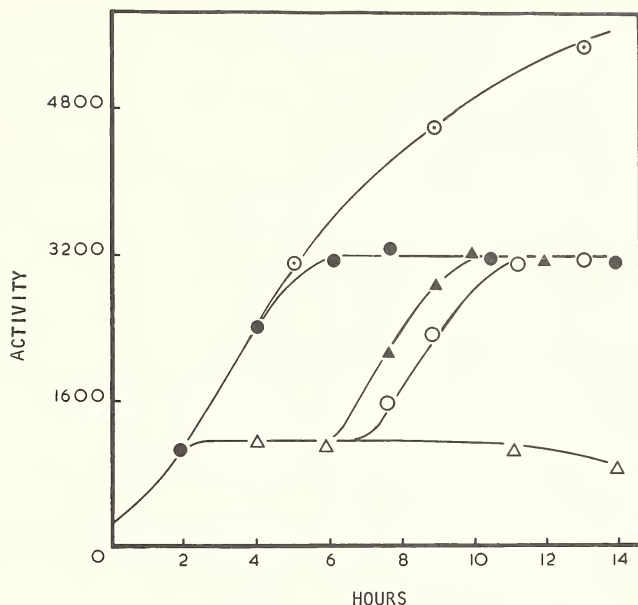
Although a number of animal viruses induce the synthesis of enzymes, investigation of control mechanisms to which these enzymes might be subjected has been confined to the poxviruses—the T-bacteriophage of the animal world. Soon after the entry of a poxvirus into its host, a decoating process occurs (1, 2) which has the aspects of an inducible enzyme system. This decoating brings about the conversion of the virus to a state in which the genome can express both its homocatalytic and heterocatalytic functions.

Poxvirus is replicated at discrete sites (viral “factories”) within the cell cytoplasm (3). Consideration of the cytoplasmic site of viral DNA replication led to a study of the enzymes involved in its replication. No unusual purines or pyrimidines whose synthesis would require novel enzymes have been detected in poxvirus DNA. The genome of poxvirus with a molecular weight of $80\text{--}160 \times 10^6$ Daltons (4) could conceivably code for several hundred different proteins of which only 20 are demonstrable by immunodiffusion techniques. Increases in enzyme activities concerned with DNA metabolism are elicited soon after infection of mammalian cells in culture, and their syntheses appear to be regulated (5, 6).

REGULATION OF ENZYME SYNTHESIS

The synthesis of the enzymes commences about 1 hour after infection, and over the next 6 hours their activities increase some tenfold. Further increase is then rapidly terminated, and the activities remain constant for many hours. Termination of kinase synthesis is certainly not a reflection of a general termination of protein synthesis (5, 6), so that there is some specificity about the switchoff of enzyme synthesis and it is reasonable to refer to it as regulation. For the enzymes of the T2-coli system and for thymidine kinase and alkaline DNase induced by poxvirus, termination of enzyme synthesis does not take place if the virus is irradiated with a suitable dose of ultraviolet light (UV) before infecting the cell (7, 8).

An advantage of animal cell systems is the readiness with which metabolic inhibitors such as actinomycin D and puromycin can be introduced into the animal cell to interrupt stages in enzyme synthesis. With these inhibitors we were able to demonstrate (text-fig. 1) that actinomycin D could simulate the UV effect, that the messenger for kinase synthesis is unusually stable, and that protein synthesis is necessary to block the translation of the kinase messenger.



TEXT-FIGURE 1.—The effect of inhibitors on the establishment of repression of kinase synthesis. (●) Kinase activity in poxvirus-infected cells; (○) kinase activity after addition of actinomycin at 2 hours; (△) kinase activity after addition of puromycin at 2 hours; (▲) kinase activity after removal of puromycin at 5½-hours; (○) kinase activity after addition of actinomycin 30 minutes before removal of puromycin.

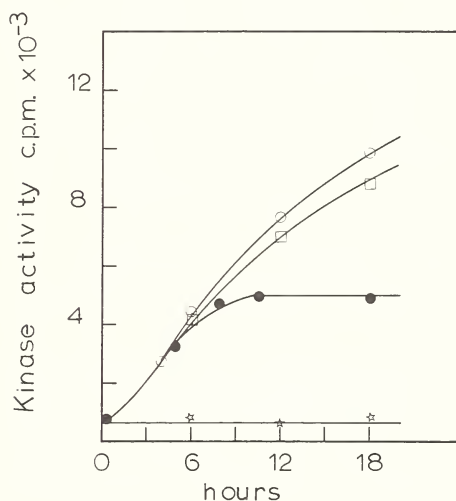
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The messenger for the virus-induced DNA polymerase is also relatively stable (9), and its switchoff is probably mediated by the same mechanism as that which regulates kinase synthesis. The synthesis of induced alkaline DNase, which has an unstable messenger, is also terminated at the same time as synthesis of kinase and polymerase. In this case, there is evidence that a regulating mechanism different from that for control of kinase is operating (6).

The Relation Between DNA Synthesis and Control of Enzyme Synthesis

For both the T2 phage and poxvirus, the UV dose that prevents the normal cutoff in synthesis of early enzymes is such that viral DNA synthesis is blocked. This led us to examine the relation between DNA synthesis and switchoff of early enzymes. No detectable breakdown of

host DNA takes place in the infected animal cell, so it is possible to inhibit viral DNA synthesis with either aminopterin or fluorodeoxyuridine (FUDR) to see if viral DNA synthesis is implicated in regulating the early enzymes. And indeed, if viral DNA synthesis is inhibited in this way, then the subsequent syntheses of kinase, polymerase, alkaline and neutral DNases are not switched off (text-fig. 2) so that "ultraviolet"-type curves are obtained (6).



TEXT-FIGURE 2.—Increase in kinase in poxvirus-infected cells in the presence or absence of fluorodeoxyuridine. Kinase activity in infected cells (●); kinase activity in cells infected in the presence of FUDR (○); kinase activity in uninfected cells (☆); kinase activity in cells infected in the presence of bromodeoxyuridine (□).

A similar effect is produced if viral DNA synthesis is allowed to proceed under conditions where bromodeoxyuridine is incorporated into progeny DNA (6). Such experiments clearly demonstrate the correlation between viral DNA synthesis and the expression of a late function—in this case the termination of early enzyme synthesis.

The relation of DNA synthesis to enzyme regulation is also shown by the events in coliforms infected with *amber* mutants of phage T4. In their nonpermissive host, a class of these mutants lacks the ability to initiate DNA synthesis; the synthesis of several early enzymes occurs but the normal cutoff of enzyme synthesis does not take place (7).

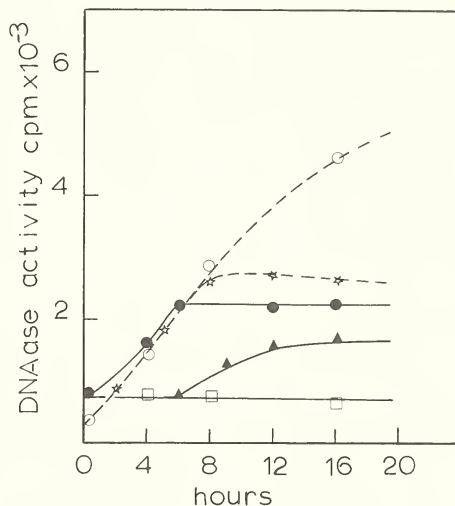
Regulation of a Late Enzyme

In the normal course of events during poxvirus replication there is not the sharp distinction between early and late functions that there is during phage replication. Early enzymes are terminated about the same time as is viral DNA synthesis, and by this time other viral antigens have appeared. However, by first blocking DNA synthesis with FUDR and then later reversing the inhibition, we can time events to closely resemble the sequence of events in T-phage-infected

coli (text-fig. 3). Thus kinase and alkaline deoxyribonuclease are synthesized in the presence of FUDR, but on removal of this inhibitor, DNA synthesis is initiated and enzyme synthesis is terminated.

TEXT-FIGURE 3.—Relation between DNA synthesis and regulation of deoxyribonucleases. Increase in acid DNase in infected cells (●); increase in acid DNase (□) and alkaline DNase (○) in the presence of FUDR; increase in acid DNase (▲) and alkaline DNase (☆) after infecting cells in the presence of FUDR and then removing FUDR 6 hours post infection.

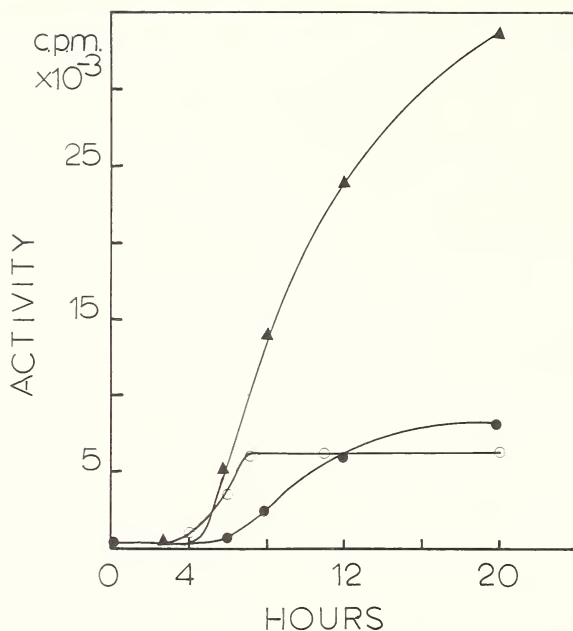
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However, in the same experiment it is evident that synthesis of acid DNase is strictly dependent on DNA synthesis. Synthesis of acid DNase commences as alkaline DNase synthesis is arrested. If DNA synthesis is permitted for but brief periods and then blocked with FUDR, acid DNase synthesis is initiated but the subsequent rate of synthesis is directly proportional to the amount of DNA synthesized. In consequence, we can consider the acid DNase as a "late" function even though in the normal system the kinetics of its synthesis are not clearly different from those of the other enzymes.

Recent studies of the acid DNase reveal that the induced activity is clearly distinct from any detectable preexisting host DNase and that it exists both in a soluble and in a particulate state. The activity can be released from the bound state by detergents. The termination of the increase in activity in the soluble state coincides with the increase in the amount of enzyme activity that can be released from the bound state (text-fig. 4). This increase continues for some 15–18 hours after infection.

It is possible that we are dealing with two different acid DNases, although studies with highly purified enzymes do not support this idea. For a virologist, the interesting feature of the enzyme is that it provides us for the first time with a readily assayable "late" pox-virus function. From preliminary studies we suspect that this enzyme in its bound state is a component of the matrix within which the virus



TEXT-FIGURE 4.—The increase in soluble and bound DNase activity in poxvirus-infected cells. Increase in soluble activity (○); increase in bound activity (●); activity releasable from the bound state by detergent (▲).

maturation. By studying both the synthesis of the enzyme and the complex to which it is bound, we may have a back-door approach to the organization of the viral factories within the cytoplasm.

From the point of view of enzyme control *per se*, it will be interesting to study the process by which the soluble enzyme becomes bound, and presumably less active *in vivo*. Certainly other membrane-bound enzymes are known, but there are few, if any, whose synthesis and change of state undergo the kinetics exhibited by the virus-induced acid DNase.

REGULATION OF ENZYME ACTIVITY

Another example of a virus-elicited enzyme whose activity rather than synthesis can undergo regulation is the thymidine kinase. The induced enzyme is quite stable if extracted from infected HeLa cells, but is completely inactivated during its extraction from virus-infected primary cultures of chick embryo fibroblasts. Soluble extracts of uninfected HeLa cells will stabilize the enzyme during its extraction from infected chick-embryo cells, and among a number of compounds tested

for their ability to act as stabilizers were the substrates thymidine and ATP.

For a number of reasons it is unlikely that these are the compounds in HeLa cell extracts that are acting as stabilizers.

Further investigating this phenomenon, we attempted to separate the stabilizing factors from thymidine kinase by gel filtration. Several interesting facts that emerged from these experiments can be summarized as follows. The enzyme is inactivated by gel filtration, but can be completely reactivated by treating it with any one of its substrates or by uninfected HeLa cell extracts. The kinetics of reactivation are strikingly different for ATP and HeLa extracts as reactivators. Inactivation by gel filtration and reactivation by ATP or HeLa extracts are accompanied by striking changes in the configuration of the enzyme. A brief outline of these findings is presented in table 1.

TABLE 1.—Change in the activity and sedimentation coefficient of thymidine kinase

Procedure	Enzyme activity*	Sedimentation coefficient†
Enzyme extracted from poxvirus-infected HeLa cells.	15,000	10–11S
Enzyme passed through Sephadex G-25.	2,000	5S
Sephadex-inactivated enzyme treated with HeLa extract for 10 minutes.	15,000	10S
Sephadex-inactivated enzyme treated with ATP for 10–60 minutes.	2,000	
Sephadex-inactivated enzyme treated with ATP for 180 minutes.	15,000	10–11S

*Enzyme activity was measured by isotopic determination of TMP formation and is expressed arbitrarily as count/min under standard conditions (5).

† Sedimentation coefficients were determined by using the moving partition cell of Yphantis and Waugh (10).

The induced kinase undergoes noncompetitive inhibition by dTTP, the distal product of the thymidine phosphorylating pathway, *in vitro*. Such inhibition is markedly influenced by the concentration of ATP present; high ATP concentrations prevent inhibition by dTTP. Large changes in the molecular weight of the Walker carcinosarcoma thymidine kinase is due to aggregation-disaggregation effects. These changes, while affecting the response to dTTP inhibition, are not accompanied by changes in enzyme activity (11). With poxvirus kinase, the reversible physical changes are presumably due to assembly and disassembly of enzyme subunits. They are accompanied by pronounced changes in enzyme activity.

DISCUSSION

The similarity between poxvirus and phage T2 in the control of early functions becomes apparent as data are accumulated for both

systems. One may well ask if there is any point in pursuing studies with the more tedious animal virus systems. The answer lies in the fact that for some experiments the animal system offers some distinct advantages, and that despite intensive investigations of the T2 system over the last 6 years one cannot describe the mechanism for the controlled sequence of events in virus replication. Our main concern with the animal virus systems is the study of virus replication as an end in itself, and the regulatory events that viral enzymes undergo are providing useful biochemical markers of stages in the replication cycle.

In the poxvirus system we have examples of the expression of both stable and unstable messengers of early functions being blocked. Whether the one mechanism blocks translation of both classes of messengers or whether there is a different mechanism for each class remains to be established.

The significance of the induction of thymidine kinase by viruses is still a biochemical enigma. Poxvirus, herpesvirus, and the tumor-inducing viruses polyoma and SV40 are all DNA viruses; and all elicit increases in kinase activity in infected cells. Because thymidine kinase undergoes inhibition by dTTP in a number of diverse systems and its synthesis is modulated during the mitotic cycle and during liver regeneration, it is assumed that the kinase is somehow directly involved in the control of DNA metabolism.

While arguments can be advanced for the selective advantage to a DNA virus of carrying the capacity to induce kinase, we really need to know if the virus actually carries the code for enzyme synthesis before one can understand the significance of kinase induction. Circumstantial evidence for and against poxvirus coding for kinase has been presented elsewhere (2, 5). In view of the obvious complexity of thymidine kinase described for poxvirus-infected cells and other mammalian cell systems, we should not overlook the possibility that the postinfection increase in thymidine kinase results from the viruses effecting configurational changes in a host-coded enzyme. The change in aspartate transcarbamylase properties by adenovirus infection is an example of a replicating DNA virus modifying the activity of an allosteric host enzyme (12).

A study of enzyme regulation in which poxvirus-infected animal cells were used has provided examples both of control of enzyme synthesis and of regulation of enzyme activity. Along with T2-infected coli, the poxvirus-infected cell serves as a useful model system for elucidating the interrelation of macromolecules in the sequential expression of a genome. Other virus-cell complexes could add another dimension to such studies for then we could compare not only those viruses that have widely differing information contents and dependence on host function but also those that are nuclear replicating and

cytoplasmic replicating. The experiments devised for the systems we now use are thus only a beginning in this area of regulation.

RESUMEN

Los desoxivirus inducen la formación de diversas enzimas relacionadas con la síntesis de ADN. Como en el caso de las enzimas inducidas por bacteriófagos, su síntesis está sujeta a regulación.

La atención se dirigió, sobre todo, a dos enzimas inducibles, la timidina cinasa y una desoxirribonucleasa. Aun cuando la síntesis de estas enzimas parece terminar casi simultáneamente, se pueden demostrar diferencias notables en el modo como se lleva a cabo esta regulación.

La síntesis de la timidina cinasa y otras dos enzimas no requiere la síntesis de ADN, aunque la terminación de la síntesis de la cinasa no dependa de la formación de ADN de la progenie viral. En contraste, la síntesis de la desoxirribonucleasa se expresa sólo por el ADN de la progenie.

Además de la regulación de la síntesis, la actividad de la timidina cinasa inducida parece estar controlada por factores celulares que cambian la configuración de la enzima. Se describen algunos datos recientes sobre el cambio de estado de la desoxirribonucleasa, de soluble a particulada.

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The Operon Concept in Bacteria and in Higher Organisms^{1,2}

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SUMMARY

The operon, defined in bacteria, is a group of contiguous genes which, on derepression, are transcribed into a single strand of messenger RNA. Both translation and transcription proceed from the operator end of the operon. Mutations to the triplets for chain termination in any of the genes of the operon cause polarity, a reduction in the activity of genes distal to the operator, as a consequence of the translation of the polygenic messenger-RNA as a unit. Operon function is controlled by two sorts of genes, operator genes (where mutations are cis dominant) and regulator genes (where mutations are recessive). In bacteria, transcription and translation are closely related. In the *lac* system, regulation appears to be directly at the level of the DNA, but in other operons, it is not known whether primary control is exerted at the gene level or by regulation of the translation of the messenger. The term "metabolite induction" is introduced to describe the regulation of certain

systems in which the product, rather than a substrate, of the first enzyme of a pathway, induces all of the enzymes of the pathway. The formation of the inducing product is usually controlled by feedback inhibition exerted by the ultimate end product. The significance of this phenomenon is discussed. In certain operons, the genes are derepressed sequentially, and the possible significance of this regulation is discussed. In animal cells and in the cells of certain eucaryote microorganisms, clusters of genes controlling either metabolic or morphological units are known. Long stretches of DNA-like RNA have been found in the nucleus. However, the average length of polyribosomes in animal cells suggests that single cistrons are the usual units of translation. It may be that the units of transcription and of translation are of different lengths, and it appears that there are distinct controls over each process.—*Nat Cancer Inst Monogr* 27: 221-234, 1967.

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RECENT WORK on protein synthesis and the genetic code has shown the main outline of the pathway by which genetic information is translated into the amino acid sequences of proteins. In contrast, the mechanisms which regulate gene expression are much less well understood. We therefore propose to discuss some aspects of gene regulation in bacteria and in higher organisms, with particular regard to the operon.

THE OPERON

One of the most useful concepts in the study of biological regulation has been the concept of the operon, which was formulated to relate three apparently different kinds of findings in bacteria:

1) The clustering on the bacterial chromosome of genes that determine the enzymes of a biochemical pathway (1).

2) Coordinate repression and derepression of the enzymes controlled by these clusters (2).

3) Specific regulatory mutations which affect the expression of the cluster of genes (3).

The crystallization and clearest exposition of the operon theory were presented in 1961 by Jacob and Monod (4). More recently, the operon has been reviewed by Ames and Martin (5) and by Ames and Hartman (6).

The generally accepted characteristics of the bacterial operon are:

(a) Control of a gene cluster. An operon consists of a cluster of genes that are activated or inactivated as a unit.

(b) Induction and repression. Certain specific small molecules (inducers or co-repressors) that are substrates or products of the pathway—the enzymes of which are coded by the genes of the operon—can cause a rapid increase (induction) or decrease (repression) in the rate of synthesis of all the enzymes of the operon.

(c) Coordinate control. To a first approximation (*see below*), the ratio of the amount of an enzyme to that of any other enzyme of the same operon is constant for given growth conditions, regardless of the extent of repression or induction (2).

(d) Polygenic message. The operon is the chromosomal unit of transcription for the formation of a molecule of messenger RNA of the same length as the operon (7, 8). Recent experiments have shown that transcription of DNA into RNA begins at the operator end of an operon (9, 10) and that translation of messenger RNA into protein also begins at the operator end of the message (6, 10, 11). Since it is known that polypeptide chains are assembled sequentially from amino- to carboxyl-terminal ends (12), it follows that genes are oriented so that the operon end of a cistron codes from the amino-terminal end

of the corresponding protein molecule. The translation of this messenger as a unit accounts for coordinate repression or induction (7, 8, 13).

(e) Polarity. The polygenic message is translated unidirectionally by ribosomes that start at the operator end. Certain mutations in the genes of an operon, besides causing a loss of function of the affected gene, also cause polarity, a relative decrease in the amounts of all enzymes specified by the genes located on the side of the mutation distal to the operator (6, 14). Polarity results from the occurrence of either "nonsense" mutations (the polypeptide-terminating triplets UAG or UAA) (15-18) or frame-shift mutations (16, 17) which give rise to nonsense triplets as a result of the shift in the reading frame during the translation of messenger RNA.

(f) Specific regulatory mutations. For most operons thus far examined, several classes of mutations have been discovered which activate the operon in the absence of the low molecular weight inducer or co-repressor usually required. These mutations are of two types: the *operator constitutive type* (O^c), which maps at the starting end of the operon and affects only contiguous genes on the same chromosome; and the *regulator gene type* (e.g., i^- in the *lac* operon), which lies on a different part of the chromosome and is recessive to the wild type. Because of these dominance relationships, it is generally concluded that the regulator genes produce a substance, the repressor, which inhibits operon function (4). The operator is thought to be the site of action of the repressor (4).

MECHANISM OF INDUCTION AND REPRESSION TRANSCRIPTIONAL CONTROL

To account for the induction and repression of operons, Jacob and Monod (4) proposed specific chemical functions for the operator and repressor. The repressor, according to their view, is an allosteric protein which binds specifically to the DNA at the operator region of the operon, thus blocking its transcription into RNA. The repressor must also combine specifically with either the inducer or the co-repressor, depending on whether the operon is inducible or repressible. In the former case, the repressor-inducer complex no longer binds the operator, whereas in the latter only the co-repressor-repressor complex combines with the operator. A mutation to O^c is thought to make the operator relatively insensitive to the repressor. Thus, in this model, operon regulation is accomplished by stimulation or inhibition of gene transcription.

A number of observations suggest that certain features of the classical operon model must be reconsidered and extended. It is our purpose in this communication to indicate certain directions in which these extensions might be made. One of the first issues is the molecular mechanism of induction and repression. The original Jacob-Monod model specifically called for a direct interaction of the repressor with DNA. This hypothesis has influenced thinking about regulation not only in bacteria but in animal cells as well. For example, the histones in higher organisms have often been considered to be equivalent of the operon-specific repressors of bacteria.

Translational Control

However, some recent observations have suggested that the primary site of repressor action may be at the level of translation, at least in some systems other than lactose. Basically, two types of theories have been presented which assign a primary role to translational regulation:

1) Regulation by transfer RNA (6, 19). It has been found that charged histidyl transfer RNA is more directly involved in repression of the histidine operon than is free histidine (20, 21). Similar findings have been made in the case of the pathway of valine biosynthesis (22). Since transfer RNA is specifically involved in translation, the possibility exists that the rate of synthesis of the enzymes of a given operon is primarily limited by the rate of translation of the messenger corresponding to the operon, rather than by the amount of that messenger, as required by the transcriptional theory. For instance, a specifically charged histidyl transfer RNA might repress operon function by blocking the translation of the histidine message, in which case the functional operator region would be at the start of the messenger RNA. Jacob and Monod (23) have argued that transcription of the operator region of the lactose system into messenger RNA does not occur. In other operons, however, there is no evidence against the operator being transcribed.

2) Regulation by folding of nascent protein chains [*e.g.*, (24, 25), etc.]. In this model, inducers or co-repressors would act as allosteric modifiers, which affect the rate of enzyme formation by altering the folding of nascent polypeptide chains. This model specifies that the operator region is part of the structural gene of a protein.

In addition to these two translational theories, enzyme induction has been attributed to stabilization of messenger RNA (26, 27). Since changes in messenger stability could easily result from changes in the

rate of messenger translation (*e.g.*, during translation messenger RNA is protected from a ribonuclease) (Logan and Singer, personal communication), this view could be considered a consequence of either translation model.

Interdependence of Transcription and Translation

At present, it is impossible to decide whether the repressor-operator interaction occurs at the level of RNA or DNA, because translation and transcription are intimately associated. For example, biochemical studies have shown that ribosomal attachment to nascent messenger RNA enhances the action of RNA polymerase (28-31) and that polar mutations decrease the amount of messenger RNA beyond the mutations, as well as prevent translation of the message (32). More obviously, primary alterations in transcription would influence the translation of the message. It seems, then, that the primary sites of inducer and repressor action in bacteria are still questionable, and that the solution may require a direct biochemical approach when it becomes possible.

Regulatory Mechanisms in Higher Organisms

In higher organisms there is evidence that specific enzyme synthesis can be regulated by both transcription and translation. Fertilization, vaccinia virus infection (33), and certain phases of steroid hormone action (34) are examples in which translation appears to be limiting.

There are also indications that direct gene activation occurs in embryonic development and hormone action. For example, specific regions of the chromosome (puffs) synthesize RNA in temporal sequence during development and under stimulation by the insect hormone ecdyson [*see* Clever (35) for review]. In addition, large portions of different chromosomes (36) or even entire chromosomes (37) can exist in an inactive state.

It seems reasonable that combinations of translational and transcriptional mechanisms of control operate in animal cells. During embryonic development, for example, there are periods when the activities of various enzymes cannot be detected in the liver, when, later, the enzymes appear (but are not inducible), and finally, when, in the adult, the enzymes can be induced by various means [*see* Knox and Greengard (38)]. This sequence suggests initial gene activation followed by other regulatory events, possibly at the translational level. Furthermore, it might be relevant that there is a large amount of RNA which turns over rapidly in the nucleus of mammalian cells but which does not appear in the cytoplasm (39, 40). This could indicate that only those messengers which can be translated are selected to appear in the cytoplasm.

MULTIVALENT CONTROL

Besides these fundamental problems of mechanism, biological considerations require the expansion of the operon concept. In the original operon proposal, the idea of interconnecting metabolic pathways with communication at the gene level was suggested. It was pointed out, for instance, that the inducer of one operon could be the repressor of another. Recent observations indicate that such considerations have to be taken very seriously, since the function of an individual operon may be regulated by many more factors than just a single inducer or co-repressor. The most obvious example is "multivalent repression," in which all the end products of a branched metabolic pathway affect a given operon. For example, Freundlich *et al.* (41) have found that all the end products of the isoleucine-valine pathway (isoleucine, valine, leucine, and pantothenate) are simultaneously required to repress the synthesis of the enzymes. These observations have been rationalized in the Jacob-Monod model by assuming that the repressor has sites for all the co-repressors and that each site must be filled before the repressor can inhibit operon activity. One could also explain the data in terms of translation control.

Catabolite repression of inducible operons must also be considered. One case of catabolite repression is the glucose effect in the *lac* system; the induction of the *lac* enzymes is unnecessary and does not occur as long as glucose is present as a carbon source. From recent experiments it appears that glucose inhibits *lac* induction by any of the following independent mechanisms (42). 1) Glucose prevents the accumulation of the inducer by interfering with its transport. 2) It activates a specific "CR" gene, which in some way inhibits the *lac* operon in a manner thought to be analogous to that of *lac* repressor. 3) It has a transient effect, which causes a momentary decrease in the rate of *lac* enzyme production even in CR⁻ organisms.

Both catabolite and multivalent repressions are physiologically sensible but complicated, and the operon model will ultimately have to explain them.

METABOLITE INDUCTION

Recently, several inducible systems which have certain unexpected characteristics in common have been reported in bacteria and higher organisms. In each, the product (rather than the substrate) of the first enzyme of a pathway induces the enzymes of the pathway. Recently, Koch, Hayashi, and Lin (43, 44), Gross (45), and Schlesinger, Scotto, and Magasanik (46) have called attention to this phenomenon,

and each group has discussed several examples. We would like to discuss several examples in the context of the present review and will refer to them as "metabolite induction."

The first enzyme of the leucine biosynthetic pathway in *Neurospora*, isopropylmalate synthetase, is controlled by leucine through both repression and feedback inhibition (45). The subsequent enzymes of the pathway, however, can only be induced by the isopropylmalate produced by the first enzyme. Therefore, a simple leucine shortage will not induce the whole pathway unless the first enzyme is functional and a sufficient amount of the precursor, ketoisovalerate, exists to insure the synthesis of leucine.

Thus, three conditions (end-product shortage, presence of precursor for the pathway, and a functioning first enzyme) are necessary to induce the remainder of the enzymes. Feedback inhibition indirectly leads to repression, since a functioning first enzyme is necessary for induction. Any pathway repressed by a metabolite end-product should be examined with this mechanism in mind.

Another example of metabolite induction is the case of glycerol utilization worked out by Lin and his associates. The first enzyme in the pathway, glycerol kinase, converts glycerol to glycerol-1-phosphate, which is the true inducer (43, 44). When glucose is present, glycerol utilization is prevented. This is accomplished because fructose 1,6-diphosphate (a metabolite of glucose) is a feedback inhibitor of the glycerol kinase (47). Therefore, feedback inhibition prevents induction by stopping the formation of the inducer.

Histidase and the other enzymes for histidine degradation are induced by urocanic acid, the product of histidase action (46). If histidine could not be converted to the inducer by the basal enzyme, induction of the pathway would not occur. If histidase, like glycerol kinase, is subject to feedback inhibition, the synthesis of the enzymes of the pathway would also be controlled by the feedback inhibitor.

The *lac* operon also shows metabolite induction. It has been shown (48, 49) that lactose is not the direct inducer because neither galactoside permease nor thiogalactoside transacetylase, the other enzymes controlled by the *lac* operon, can be induced by lactose if the organism is genetically unable to make β -galactosidase. When the organism is provided with lactose, the basal galactosidase transfers the galactosyl residue to an acceptor, such as glycerol, to form the true inducer (49). This mechanism prevents the induction of permease and transacetylase, as well as a nonfunctional galactosidase if lactose cannot be used. Possibly, metabolites of glucose or galactose could act as feedback inhibitors of β -galactosidase. Thus, the metabolites from a number of pathways could affect the regulation of the *lac* operon.

KINETIC CONTROL OF OPERON FUNCTION

Another area of research which may result in the expansion of the operon model is the kinetic study of the induction of several enzymes (or their messengers) from polygenic operons. The genes in three operons—lactose, histidine, and tryptophan—can be expressed sequentially, with the genes closest to the operator being expressed earlier than those further from it. Direct measurement of the messenger of the tryptophan operon (32) and indirect observations in the lactose system (10) have shown that the genes are transcribed sequentially into messenger RNA, starting at the operator end of the operon. The sequential appearance of the enzymes of the histidine operon may also be due to sequential transcription of the operon (11).

Several findings in both the lactose and histidine systems suggest that additional regulatory mechanisms control the time course of induction of an entire operon. Berberich *et al.* (11), for example, have shown that the appearance of the histidine biosynthetic enzymes may be either sequential or, in certain instances, simultaneous. Sequential derepression occurs in mutants which can produce amino-imidazole carboxamide ribonucleotide (AICAR), an intermediate in adenine biosynthesis and a side product of histidine biosynthesis. Simultaneous derepression occurs in organisms which can not produce this intermediate. Furthermore, sequential derepression can be converted to simultaneous derepression by adding adenine. Simultaneous derepression can be converted to sequential derepression by adding amino-imidazole carboxamide ribonucleoside.

At the present time, the mechanistic differences underlying sequential versus simultaneous derepression are not known, but the existence of the phenomenon (together with its modification by a specific metabolite, adenine) suggests that the control of the kinetics of derepression may have physiological importance (11).

Recent experiments on the lactose system have shown that β -galactosidase is synthesized while part of the polygenic messenger RNA is still attached to the DNA (10). Furthermore, if translation of the galactosidase portion of the messenger is restricted, transcription of the remainder of the operon is slowed.

These observations on both the histidine and lactose systems show that complicated controls exist which regulate the kinetics of induction and may in certain circumstances prevent the expression of "later" genes of an operon, even after "earlier" genes have been activated.

The biological significance of these kinetic controls is not known; however, in the histidine system one can make an argument for the physiological utility of sequential derepression of the histidine enzymes in the presence of a false feedback inhibitor. If such a compound, thiazolealanine, is added to *Salmonella* it inhibits the first

enzyme of histidine biosynthesis (50, 51). The cell then responds by sequential derepression, each enzyme coming up about 3 minutes after the preceding one (11). Since the only bottleneck in making histidine is the amount of functional first enzyme, the fact that the first enzyme is derepressed 3 minutes before the next of the other 9 enzymes of the pathway enables the cell to first circumvent the feedback block with a minimum of protein synthesis.

Thus, the momentary noncoordinacy due to sequential derepression would be an economy for the organism. In fact, in a considerable number of cases (5) the first enzyme of a pathway is coded for by the gene at the beginning (operator end) of the operon. A second interesting implication is that mechanisms regulating the sequential activation of genes within an operon in bacteria could help to explain the sequential gene activation required for the development of differentiated organisms (35).

THE QUESTION OF OPERONS IN HIGHER ORGANISMS

The operon in bacteria is defined as the unit of genetic transcription. All the genes of a given operon are transcribed into a single polygenic strand of messenger RNA. Many of the properties thought of as typical of operons, such as polarity, derive from the fact that operons are also the units of translation as discussed in the first section entitled "The Operon." In higher organisms, however, the units of transcription and of translation are not always of the same length. Since the chromosomal DNA is confined to the nucleus and protein synthesis occurs largely in the cytoplasm, the processes of transcription and translation are physically separated. If the term "operon" is restricted to the segment of DNA transcribed as a unit, some confusion arises as to the definition of messenger RNA. Perhaps "messenger RNA" should refer to the RNA which is actually translated, and a term such as "nascent messenger RNA" be used to describe RNA which is synthesized but not necessarily translated.

Direct evidence has been presented which shows that nascent messenger RNA in the nucleus may be much larger and turn over more rapidly than messenger RNA in the cytoplasm. For many years Harris (39) has pointed out that a large fraction of newly synthesized nuclear RNA never reaches the cytoplasm [*see also* (40)]. More recently, other investigators (52-54) have isolated rapidly labeled RNA of very high molecular weight from the nuclei of animal cells. In agreement with the observations of Harris, only a fraction of this RNA appears in the cytoplasm; in addition, this cytoplasmic messenger RNA is of much lower molecular weight than the nuclear RNA.

The operon concept as developed in bacteria is not easily adapted to animal systems. In bacteria the operon was originally defined as a regulatory unit, which is a useful definition, even though it has not been established whether the operon is regulated during transcription or translation. In animal cells, however, it is not clear to which aspects of the complex regulatory mechanisms the term "operon" should refer. The transcriptional and translational units are different from each other in animal cells. Transcriptional regulation in animal cells occurs at the level of the activation or inactivation of entire chromosomes, large chromosomal segments, and possibly smaller units. Translational control has also been shown. Selection of specific messengers, perhaps by their stabilization, could also be regulated because more of the genome is transcribed than actually functions as messenger RNA.

Biochemical arguments for the existence of operons in animal cells have been based on coordinate rises and falls in enzyme levels, but this type of evidence is not too helpful. In animal cells steady-state enzyme concentrations are determined by rates of enzyme inactivation and enzyme synthesis, and it is clearly only the latter process which would be useful in determining coordinate synthesis. Even if coordinate induction of several proteins could be shown, genetic linkage would not be indicated, since in bacteria, unlinked but functionally related genes may respond together to a common inducer or co-repressor. Finally, even if coordinacy and genetic linkage could be demonstrated, the original unit of transcription might have been much larger.

Despite the difficulty of transferring the idea of an operon, it is important to evaluate the role of gene clusters in higher organisms. In the operon, genetic linkage obviously allows functionally related genes to be activated by a common mechanism. However, even in bacteria all the genes for certain metabolic pathways are not in a single cluster; in the arginine and cysteine systems, for example, several clusters occur for each pathway.

Higher on the evolutionary scale, the linkage of genes governing biochemical pathways becomes less common. For example, many of the pathways in *E. coli* and *S. typhimurium* for which all the genes are clustered do not show complete genetic linkage in *Neurospora* or yeast. As an extreme example, the cistrons which control the α - and β -chains of hemoglobin in mammals segregate independently.

Nevertheless, several examples of operon-like behavior have been discovered in eucaryotes. The 3 genes governing the enzymes of the galactose pathway in yeast are clustered and controlled together by several unlinked regulatory elements (55). Three genes of the histidine biosynthetic pathway in yeast are clustered (56), as are 5 genes of the aromatic pathways in *Neurospora* (57).

Even higher on the evolutionary scale are examples of the clustering of functionally related genes, but the relations between the functions of the genes within the cluster are often not metabolic, but developmental and morphological (*e.g.*, the genes concerned with tail structure in the mouse are linked). Lewis (58) has recently reviewed the significance of gene clustering in higher organisms, giving many examples [*see also* (57)]. The phenomenon of puffing, where fairly large sections of the chromosome are activated together, may indicate the expression of these functionally related clusters.

To summarize, the operon concept has been valuable in explaining regulatory phenomena in bacteria. In higher organisms the functional organization is much more complex, and regulation can occur at all levels, from the inactivation of entire chromosomes to translational control of specific enzyme synthesis. Therefore, the operon can form only the nucleus for the explanation of these complicated phenomena.

RESUMEN

El operón, definido en bacterias, en un grupo de genes contiguos los cuales, durante la desrepresión, son transcritos a una sola banda de RNA mensajero. Tanto la traducción como la transcripción se llevan a cabo a partir del extremo operador del operón. Mutaciones en los tripletes para terminación de cadena en cualquiera de los genes del operón causan polaridad; una reducción en la actividad de genes distales al operador, como consecuencia de la traducción del RNA mensajero poligénico como una unidad. La función del operón es controlada por dos clases de genes, genes operadores, mutaciones en las cuales son *cis* dominantes y genes reguladores en donde las mutaciones son recesivas.

En bacterias la transcripción y la traducción están intimamente relacionadas. En el sistema *lac* la regulación parece estar directamente a nivel del DNA pero en otros operones, no se conoce si el control primario es ejercido al nivel del gene o por traducción reguladora del mensajero.

El termino "inducción de metabolitos" se ha introducido para describir la regulación de ciertos sistemas en los cuales el producto, más que un sustrato de la primera enzima de una vía, induce a todas las enzimas de la misma. La formación del producto inductor está generalmente controlada por retroinhibición ejercida por el producto final primario. El significado de este fenómeno es discutido. En ciertos operones, los genes son desreprimidos secuencialmente y el posible significado de esta regulación se discute.

En células animales y en células de ciertos microorganismos eucariotes, se conocen grupos de genes que controlan unidades metabólicas o unidades morfológicas. En el núcleo se han encontrado largas tiras de RNA similes a DNA. Sin embargo, la longitud promedio de los poli-ribosomas en células animales sugirió que los cistrones sencillos son las unidades comunes de traducción. Puede ser que las unidades de transcripción y de traducción sean de longitudes diferentes, y parece que existen controles distintos para cada proceso.

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Changes in RNA Metabolism During the Development of *Rhynchosciara angelae* ^{1,2,3}

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SUMMARY

Both DNA and RNA contents from the salivary glands of *Rhynchosciara angelae* increase up to the stage at which the giant chromosomal puffs appear in their chromosomes and then decrease. Using pulse labeling and centrifugation in sucrose density gradients, we determined that the principal class of RNA which is most rapidly synthesized before the appearance of the giant puffs is a 35S RNA. This RNA is subsequently transformed into ribo-

somal RNA. Subsequent to the appearance of the large puffs in the chromosomes, one observes an almost complete inhibition of the synthesis of the ribosomal precursor. The data are consistent with the hypothesis that the nucleic acids produced at the chromosomal puffs are important in the regulation of RNA metabolism in these cells.—Nat Cancer Inst Monogr 27: 235–242, 1967.

DURING DEVELOPMENT, the polytene chromosomes of several species of *Diptera* show characteristic puffing patterns that are tissue-specific (1, 2). Studies by various investigators have revealed that the puffs represent sites of intensive RNA synthesis (3–5), and in *Rhynchosciara* and *Sciara* it is strongly indicated that a disproportionate, localized DNA increase takes place at least in some puffs (5–7). These conclusions rest on histological and autoradiographic studies, and though important developments are still to be expected

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² See Discussion of this paper conducted by Dr. E. L. Tatum, p. 243.

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⁴ We are grateful to C. R. Lopez and L. Foresti, who collaborated in the initial stages of this work, to R. Meneghini, H. A. Armelin, H. Tamaki, and S. R. dos Santos for help in several experiments, and to Mrs. H. S. Lafayette for help in preparation of the manuscript.

from further application of these techniques, it is evident their very nature limits the information that can be obtained.

It is important to determine the species of nucleic acids produced at the puffs. *A priori*, this information could be obtained by isolating nucleic acids from a few homologous puffs and then determining their characteristics. An attempt in this direction has been made by Edström and Beermann (8), but the very small amount of material obtained has greatly hindered this line of approach. An alternative approach consists of isolating nucleic acids *en masse* and attempting to establish correlations between alternations of their biological, chemical, and physicochemical properties and the appearance of the puffs.

This paper relates experiments carried out along these lines with *Rhynchosciara angelae*, a species which because of its biological peculiarities—particularly the synchrony of development within a larval group—constitutes a very suitable material for studies of chromosomal physiology.

MATERIALS AND METHODS

Animals.—*Rhynchosciara angelae* were raised under laboratory conditions as previously described (9). The physiological age of the larvae was determined according to the method of Guaraciaba and Foresti (10), which is based on the known sequence of alternations in larval behavior and chromosomal morphology during development. Incorporation studies were made at stage 3, and early and late stage 5 (prepupa) as defined by Guaraciaba and Foresti. These stages correspond, respectively, to times before, during, and after the appearance of the giant chromosomal puffs.

RNA extraction.—Approximately 70 salivary glands were lysed in 0.01 M Tris, pH 7.4, containing 0.001 M $MgCl_2$ and 0.5% sodium dodecylsulfate. The suspension was deproteinized twice with 80% phenol, and then with chloroform-isoamyl alcohol (24:1). The final aqueous phase was made 0.1 M to NaCl, and the nucleic acid precipitated by addition of 2 volumes of alcohol. After standing at $-15^\circ C$ for at least 2 hours, the nucleic acids were collected by centrifugation and dissolved in 0.01 M Tris, pH 7.4, and 0.001 M $MgCl_2$.

This method results in recovery of 90% of the RNA initially present in the glands and 70% of the radioactivity incorporated as RNA. The preparations present a sedimentation profile in sucrose gradients similar to those observed in the other animal cells, and they do not show optically visible amounts of "heavy RNA" previously reported to occur when high ionic strength prevails during the extraction procedure (11).

Isotope administration.—This was made by 2 μ l injections of ^3H -uridine (7.2 c/mmM, 1 mc/ml, New England Nuclear Corporation, Boston, Mass.) into the body cavity of larvae previously anesthetized by ether.

Sedimentation analysis.—This analysis was at 2 C for 5 hours at 32,000 rpm in an SW39 head of the Spinco model L centrifuge in 5–30% sucrose gradients with 0.01 M Tris, pH 7.4, and 0.001 M MgCl_2 . Thirteen-drop fractions were collected, and OD measurements made after addition of 1 ml of water to each tube. Sedimentation constants were calculated with reference to the main ribosomal RNA component as 28S.

Radioactivity assay.—To each tube from the sedimentation analysis was added 100 μ g of serum albumin as carrier, and the RNA was collected on Millipore membranes after cold trichloroacetic acid precipitation. The membranes were dried and placed in vials filled with 10 ml of toluene scintillation solution; they were counted after remaining in the freezing compartment of scintillation Tricarb spectrometer for at least 5 hours.

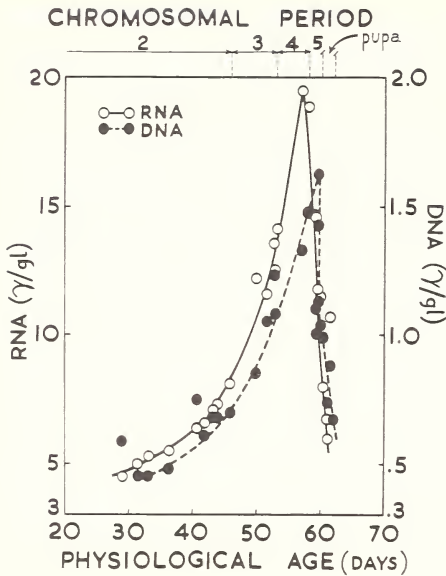
RNA determination.—The RNA content of the salivary glands during development was determined by a modification of the method of Scott *et al.* (12), in which RNA hydrolysis by ribonuclease was substituted for alkaline hydrolysis, and the volumes of reagents were scaled-up 20-fold.

RESULTS

Our first attempt to correlate important changes in nucleic acid metabolism in salivary glands with chromosomal puffs consisted of a determination of the levels of nucleic acids in these organs throughout the larval development. The results are given in text-figure 1, where the levels of both DNA and RNA appear to reach maximums corresponding to definite periods (late stage 4 and early stage 5) and coinciding in time with the appearance of many large puffs in the chromosomes.

The results could be anticipated from morphological observations; for the imago has no salivary glands, and the observed drop in the levels of nucleic acids must represent a preparation for, or part of, the histolytic process which occurs during the pupal life.

To obtain more information about this phenomenon, the pattern of synthesis of RNA was studied. To this end a large group of female larvae was divided into 3 subgroups of approximately 100 larvae. At characteristic periods of larval development (namely, before, during, and after the appearance of the large puffs), each larvae was injected with ^3H -uridine. Half the larvae in each group were killed 40 minutes



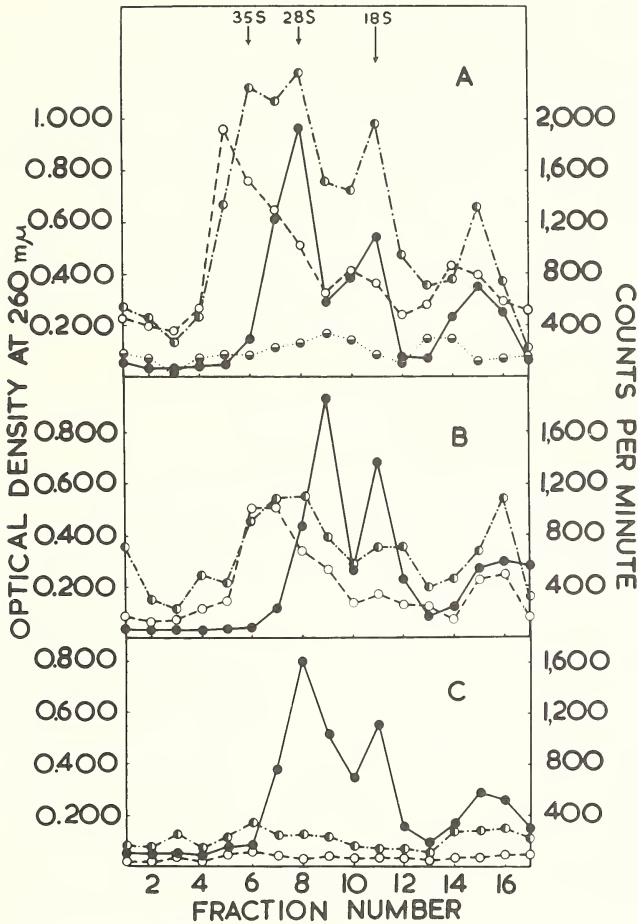
TEXT-FIGURE 1.—RNA and DNA content in salivary glands of *Rhynchosciara angelae* at several ages. Each point represents a determination with 20 salivary glands. Physiological age of a larva is established according to Guaraciaba and Foresti (10) *i.e.*, by comparison of its behavior and salivary chromosome morphology with those of a standard group of known chronological age.

later and the other half after 80 minutes. The RNA was extracted and analyzed by centrifugation in sucrose density gradients.

The experiment carried out with larvae at the developmental stage prior to appearance of large puffs in the salivary gland chromosomes is shown in text-figure 2A. Here the following points may be observed. 1) Incorporation during 40 minutes results in labeling of an RNA heavier than the 28S RNA and having a sedimentation constant of approximately 35S. There is also incorporation in other places in the gradient, with peaks being observed at 18S and 4S regions. The specific activity of the 18S RNA is about twice as large as that in the region of 28S RNA. 2) Incorporation during 80 minutes leads to labeling of all classes of RNA present in the profile observed by OD measurement, as well as of the 35S mentioned above. 3) The radioactivity is actually incorporated into RNA, for treatment with RNase releases most of this radioactivity into an acid-soluble form.

When the same experiment is carried out with larvae of the age at which the giant puffs are present in the salivary chromosomes (text-fig. 2B), the results differ from those observed above. In general, the specific activities are lower, and not much difference is observed between incorporations for 40 and 80 minutes.

The data in text-figure 2C show the results obtained with larvae at the end of the fifth developmental stage, when the giant puffs have regressed. In this case practically no incorporation was observed; it thus seems that the inhibitory processes, already apparent at the stage where the puffs are present (text-fig. 2B), reach their maximum intensity at this age.



TEXT-FIGURE 2.—Sedimentation analysis of RNA labeled with ^3H -uridine obtained from the salivary glands of *Rhynchosciara angelae* larvae at three different developmental stages. A: Before appearance of giant puffs in salivary chromosomes (age 50 days). B: When many giant puffs are present (age 57 days). C: After the giant puffs have regressed (age 59 days). For experimental details see "Materials and Methods." Optical density profiles in each timed experiment were similar and only one is shown in each case: ●—●, optical density at 260 $\text{m}\mu$; ○—○, radioactivity incorporated in 40 minutes; ○—○, radioactivity incorporated in 80 minutes; ○—○, radioactivity incorporated in 80 minutes remaining after treatment with ribonuclease.

It is impossible to decide, on the basis of the present experiments, if the observed inhibition is at the level of synthesis or results from a rapid destruction of the rapidly labeled RNA by degradative enzymes which might increase in late larval life. However, it has been possible to demonstrate that such degradative enzymes are not operative dur-

ing the RNA extraction process. Thus, uniformly labeled RNA from actively incorporating salivary glands was found to present similar sedimentation profiles when such salivary glands were extracted in the presence or absence of nonradioactive salivary glands from larvae in which the puffs had already regressed. The only difference obtained was in specific activity, which can be easily explained by the dilution of the isotope.

A distinction among the two possibilities mentioned is possible on the basis of the determination of the levels of enzymes involved in RNA synthesis and degradation. Such determinations are presently being undertaken at this laboratory.

DISCUSSION

The results presented in this paper indicate a correlation in the occurrence of important molecular events in the cells and of morphological alterations in the chromosomes. A further definition of this system will provide a rather direct approach to the problem of gene function and regulation in animal cells.

The incorporation experiments (text-fig. 2) approximate the conditions of pulse chase experiments. In this case, however, the chase of the isotope is realized by the pool of precursors normally existing in the cells. Under these conditions, the rapidly labeled material matures into RNA having the sedimentation characteristics of ribosomal RNA. Actinomycin-D chase experiments (13) also support the conclusion that most of the rapidly labeled RNA is a ribosomal precursor. This is in accord with what has been determined in growing animal cells (14); the salivary glands up to the third stage of development (10) are also growing organs.

It is interesting that in the early stage of larval development, incorporation during 40 minutes leads to the formation of a 35S RNA. In HeLa cells the first RNA forms sediments at 45S; it is then transformed into 35S RNA, and only then matures to ribosomal RNA (14). Data accumulated in several laboratories indicate that the nucleolus is the place of ribosomal RNA synthesis (15, 16). The cells of the salivary glands of *Rhynchosciara* have no typical nucleolus, and this might be correlated with the normal nonexistence of 45S RNA in these organisms.

The data indicate that, in the 40-minute incorporation experiments, one never observes a distinct class of molecules sedimenting at 28S, whereas the 18S class is always very distinct and has a specific activity that is always higher than that observed in the 28S region. This indicates that the processes leading to the formation of two classes

of ribosomal RNA have different velocities, as has been reported for rat liver (17), HeLa cells (18), and bacteria (19).

Possibly, the control of RNA synthesis depends on the concentration of amino acids available to the cells, as has been found with bacteria (20, 21). In insects a large fraction of the hemolymph osmolarity is made up by the amino acids it contains, and *Rhynchosciara* is no exception (22). When the larvae enclose themselves in the collective puparium, they cease to feed, and a drop in the amino acid content of the hemolymph is to be expected. If this happens, one can also expect a discharge of the soluble RNA, and such discharged molecules have been implicated as inhibitors of RNA synthesis (20).

RESUMEN

Tanto el contenido de ADN como el de ARN de las glándulas salivales de *Rhynchosciara angelae* aumenta hasta el estadio en el que los "puffs" cromosómicos gigantes aparecen en sus cromosomas y después disminuyen.

Por medio del empleo de marcado de pulsos y centrifugación en gradiente de sacarosa, logramos determinar que la clase principal de ARN que es sintetizada más rápidamente antes de la aparición de los "puffs" gigantes es la de 35S. Este ARN es ulteriormente transformado en ARN ribosomal.

Después de la aparición de los grandes "puffs" en los cromosomas se observa una casi completa inhibición de la síntesis del precursor ribosómico.

Estos datos están de acuerdo con la hipótesis de que los ácidos nucleicos producidos en los "puffs" cromosómicos pueden tener importancia en la regulación del metabolismo del ARN en estas células.

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DISCUSSION ¹

Discussor, DR. E. L. TATUM, The Rockefeller University, New York, New York

I WANT first to express, on behalf of the participants from outside of Mexico, and particularly from the United States, our most hearty thanks to the organizers of this excellent Symposium and for the hospitality shown us.

The assignment to discuss this session is rather difficult, not as much because it is somewhat heterogeneous, but because of its broad scope and the diverse organisms discussed. I will try, however, to put this session into historical perspective, as I see it.

Interest in the regulation of metabolism is a rather recent development. Biochemical genetics started with the broader aspects of an organism, linking gene to phenotype. As more was learned, it then became gene to enzyme to phenotype. With the recognition of the role of RNA, especially messenger RNA, interest focused on gene to mRNA transcription, and on mRNA translation into enzyme protein. The regulation of metabolism within an organism or within a cell was soon recognized to take place at these various levels. The operon-operator concept was originally concerned with control at the level of translation of DNA to RNA. As discussed by Dr. Ames for the enzymes of histidine biosynthesis, however, we must also consider temporal control at the level of translation of a polycistronic messenger into proteins. A more sophisticated approach is now also being applied to the regulation of enzyme structure and activity at the purely enzyme level, as illustrated by Dr. Koshland's talk. The effects on activity of binding of ligands and of associations and interrelationships of enzyme subunits represent the sophisticated application of physical-chemical techniques to determine the conformational structure of enzyme molecules under various conditions. Thus, at these different molecular levels, we are approaching the goal of under-

¹ Of articles by E. Volkin; B. R. McAuslan; Gordon M. Tomkins and Bruce N. Ames; and F. J. S. Lara and F. M. Hollander.

standing control processes as they affect the metabolism and structure of DNA, RNA, and enzymes.

I want secondly to emphasize the roles of viruses, first bacterial viruses, discussed by Dr. Volkin, and now increasingly, animal viruses, discussed by Dr. McAuslan, in the development of the whole concept of mRNA, and of the interrelationships between viral and host-cell regulating mechanisms. Just as the bacterial virus system gives insight into regulatory processes in the bacterial host cell, so the animal virus system will give increasing insight into the more complicated regulatory mechanisms in animal cells. The similarities between regulatory mechanisms in bacterial and animal virus systems are indeed striking, taking into account the greater complexity of the animal cell, which is a necessary function of the complexity of multicellular differentiation.

This touches on the next big step in the biochemistry of regulation—that responsible for development and differentiation. A most promising subject for such an extension appears to be *Rhynchosciara*, in which, as Dr. Lara described, chromosome and gene activity with message transcription and translation can be directly examined and correlated.

There were, to me at least, several particularly interesting conclusions to be made from the papers in this session. First, it would seem that probably every variation and modification in regulatory control of metabolism which we can imagine will be found in one organism or system or another. Nature is most ingenious, imaginative, and sometimes to us, incomprehensible. We must ask the right question to get a true answer. For example, as between gene regulation at the transcription level versus the translation level, it is becoming clear that these two processes may be only conceptually separated, and may actually be functionally interrelated. It would appear, also, that some of the apparent discrepancies in the applicability of the operon concept to bacterial and animal cells may be resolvable with the increased emphasis on the translational process through the mediation of transfer RNA. The varied function of tRNA with mutation and regulation seems a very promising new area of interest.

Finally, a still more general comment on the relation of this session to others in this Symposium. It is gratifying to me to see the study of regulation of metabolism retracing the steps of biochemical genetics, and being extended from the regulation of enzyme activity *per se* back to regulation of gene transcription and translation.

In answering questions (Drs. Reissig, Cabrera) regarding the oligonucleotide attaching enzyme, Dr. Volkin state that this enzyme, discovered and studied by Dr. Mead, is detectable in uninfected *E. coli*

cells, but appears to be present at surprisingly higher levels in recombination negative mutants of *E. coli* than in the normal strain B. Single-stranded DNA is essentially inactive as acceptor, and labeled nucleotides appear to be added terminally. It is not yet known whether nucleotides are attached to one or both DNA strands.

In answer to a question (Dr. Estrada) regarding immunological evidence for the appearance of new virus-induced proteins, Dr. Volkin briefly summarized experiments using antisera against infected and uninfected *E. coli*, which indicate that infected cells contain proteins that appear to be neither bacterial nor phage structural.

Dr. McAuslan, answering a question (Dr. Toledano) regarding the pox-virus-induced thymidine kinase, pointed out that reactivation of inactivated kinase by ATP and by HeLa cell extracts appears to involve different mechanisms. At least the kinetics of reactivation are different. HeLa reactivation is complete in 10 minutes, while ATP reactivation involves an appreciable lag and a noncatalytic kinetics. There is some evidence that something other than ATP itself is involved, possibly acting with other factor(s) in the HeLa extract.

The session ended with a cartoon slide provided by Dr. Ames of a Persian-rug maker. Dr. Ames pointed out the moral—that, in research, as in rug designing, a great deal of thought and effort can be invested, only to find that the project had already been done by someone else.

WEDNESDAY AFTERNOON

Chairman: Edmundo Calva

Regulation of Active Transport^{1,2,3}

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SUMMARY

Active transport mechanisms are logical sites for metabolic regulation, owing to their positions at the beginnings of metabolic pathways and to their requirements for energy. Their regulation by induction and repression has been reported in bacteria. Regulation by feedback inhibition of transport activity has been proposed in two cases. Feedback inhibition of transport is difficult to investigate with intact

cells. Active transport of sulfate is regulated by both repression and inhibition and is being investigated at the subcellular level. A sulfate-binding protein has been isolated. Its properties *in vitro* and its relation to active transport are discussed. A tentative model for sulfate transport is presented in the light of our results.—*Nat Cancer Inst Monogr* 27: 249-257, 1967.

ACTIVE TRANSPORT of compounds into living cells has been known for a long time (1). It requires energy and is the first step in the metabolism of many nutrients. Control of transport saves energy used by the process itself and limits the rates of subsequent reactions (2, 3). It is not surprising that we see the same control mechanisms in active transport as are seen in enzymatic reactions. However, very little has been done toward investigating these regulatory processes, because we do not understand the biochemistry of active transport and are not readily able to investigate the controlling elements within intact cells.

The object of this brief communication is, first of all, to describe some background and then some recent experiments on the function

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² See Discussion of this paper conducted by Dr. R. L. Metzenberg, p. 297.

³ This work was supported by Public Health Service grant AI-04409 from the National Institute of Allergy and Infectious Diseases.

⁴ Many of the experiments presented here were performed by my colleagues—Dr. J. Dreyfuss, L. S. Prestidge, M. B. Whipple, and S. Karipides.

and regulation of a sulfate transport system. We shall restrict ourselves to bacteria, since they provide some of the more striking examples of regulation of transport activity (4).

INDUCTION AND REPRESSION OF ACTIVE TRANSPORT SYSTEMS

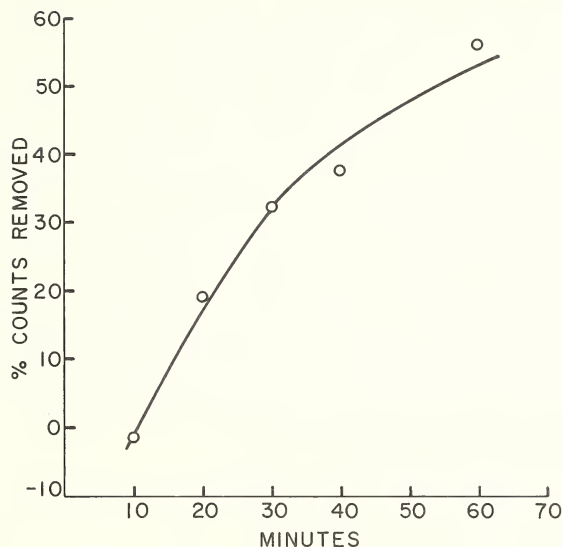
At least two types of regulation of biochemical reactions are now recognized. One is the regulation of the formation of enzymes, which determines the number of enzyme molecules (5). The other is the regulation of activity of enzyme molecules by processes such as activation and end-product inhibition (6, 7). Both of these types have been observed in a few active transport systems.

Modern development in bacterial active transport commenced with the discovery of an active transport system for β -thiogalactosides in *Escherichia coli* (8). The name "permease" was suggested for such systems to show their similarity to inducible enzymes. This term has utility in that it indicates succinctly the type of system under discussion. One can account for the kinetics of β -galactoside permease activity by postulating the existence of a carrier similar to an enzyme which combines with the β -thiogalactoside and carries it into the cell by an energy-dependent reaction. This inflow is counterbalanced by a specific, nonenergy-requiring outflow (4). Kinetics of this type have been studied extensively in a variety of systems and have led to theoretical models (1). An example will be given later.

The β -galactoside permease is inducible and its synthesis is controlled by the same genetic-cytoplasmic system as controls the formation of the enzyme β -galactosidase (5). The two structural genes form parts of the same operon, a unit of genetic control. Thus, the mechanisms for control of formation of β -galactoside permease and β -galactosidase are identical.

Repression of the system for active transport of inorganic sulfate into *Salmonella typhimurium* has been observed (9). A mutant, *cys CD-519*, which cannot metabolize sulfate was used in most experiments. Bacteria grown on a good sulfur source (such as cysteine) are repressed and do not actively transport sulfate. Transport is rapidly and markedly derepressed when the bacteria are shifted from cysteine to djenkolate-containing medium (text-fig. 1). Djenkolate, a poor sulfur source, creates a low internal pool of sulfur-containing compounds and thus permits derepression.

Undoubtedly, the whole spectrum of regulations already demonstrated for enzyme synthesis will be discovered for active transport systems. For example, induction of an exit mechanism for galactose has been reported (3). Several permeases in the same cell can trans-



TEXT-FIGURE 1.—Derepression of sulfate transport. Mutant *cys CD-519* was grown in the presence of L-cysteine, was washed once, and then grown in medium with L-djenkolate. At intervals, samples were taken for assay for the ability of a dense bacterial suspension to remove radioactive sulfate from the medium after 1 minute of incubation. The bacteria were separated by filtration through a Millipore filter covered with a layer of celite to prevent clogging (9).

port one substance (10, 11). Although, like enzymes, they might simply have overlapping specificities, the permeases might also cooperate for control under different environmental conditions, as do regulatory enzymes (7).

Regulation of the quantity of permease has the obvious advantage of preventing the expenditure of energy and building blocks for the formation of unused transport systems. More energy is saved when they do not operate.

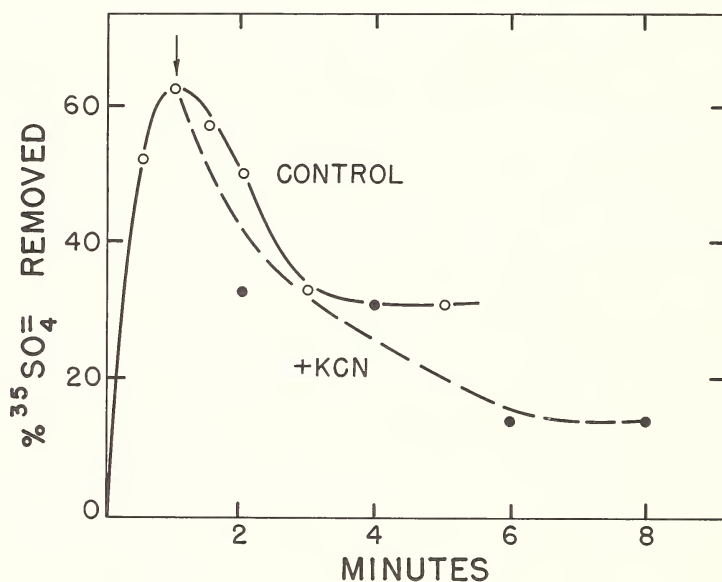
INHIBITION OF ACTIVE TRANSPORT

In feedback or end-product inhibition, a compound inhibits the activity of an early enzyme involved in its own metabolic pathway, thus providing an effective regulation of that pathway. One might anticipate that a metabolic intermediate could control its pathway by inhibiting entry into the cell of its initial nutrient. Only two examples of this type of regulation have been reported.

The transport of glucose into muscle (2) and *S. typhimurium* and *E. coli* (12) was actually stimulated by inhibitors of energy produc-

tion, such as azide or cyanide. This result suggests that glucose transport is inhibited by high-energy metabolites which accumulate during normal metabolism of the glucose. When the formation of these metabolites is inhibited, glucose transport is greater. The inhibition must occur not only in enhanced exit but to some extent in the entry mechanism, since the concentration of substrate required for saturation of entry is changed (13).

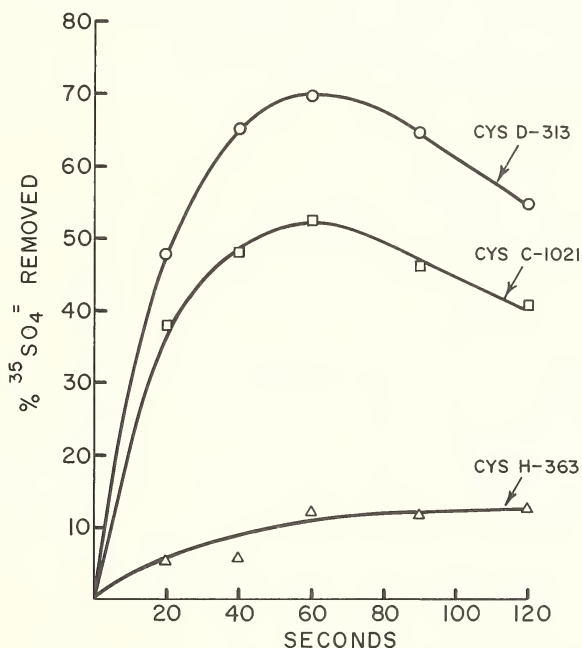
Sulfate transport appears to be regulated by feedback inhibition (14). The kinetics of this process are peculiar in that the initial inflow of sulfate is followed by an outflow which finally comes to an equilibrium at a concentration intermediate between the maximum and the concentration in the medium (text-fig. 2). This overshoot suggests that an inhibition mechanism is gradually activated by intracellular sulfate.



TEXT-FIGURE 2.—Sulfate transport and the effect of cyanide. Mutant *cys CD-519* was grown on djenkolate. Transport was determined as in text-figure 1, after various times of incubation. At 1 minute, 0.02 M KCN was added to the experimental samples.

Inward active or passive flow of sulfate requires energy (9). Outflow does not, as shown by the escape of sulfate in the presence of cyanide (text-fig. 2). The overshoot occurs only when the bacteria have an adequate energy supply and a fairly high external concentration of sulfate (5×10^{-5} M). These results suggest that a high-energy sulfate intermediate accumulates within the bacteria and reduces the

rate of inflow relative to outflow. In agreement with this suggestion, the transport system of *cys H* mutants is not nearly so active as that of *cys C* or *D* mutants (text-fig. 3). *Cys H* mutants are blocked in the further metabolism of 3'-phosphoadenosine-5'-phosphosulfate (PAPS), a high-energy intermediate in the pathway of sulfate metabolism. An internal pool of PAPS is probably the main difference between this mutant and others. PAPS might inhibit sulfate transport in the same fashion as some unknown intermediate of transport in the *cys CD* mutant.



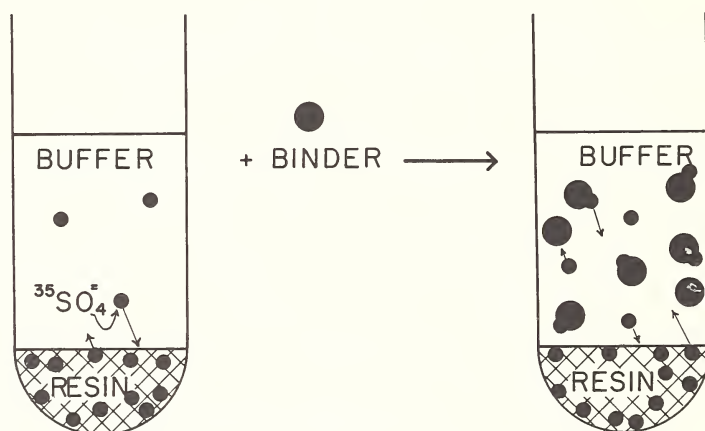
TEXT-FIGURE 3.—Sulfate transport by several mutants. The experimental design was the same as in text-figure 2.

Recently two other reports of feedback inhibition of transport have appeared: amino acid transport by *Streptomyces hydrogenans* (15) and adenine transport by *Schizosaccharomyces pombe* (16).

Further understanding of transport requires other approaches, such as identification and characterization of individual parts of the system. Progress in this direction has been made by the isolation of a part of the sulfate transport system which we refer to as "Binder" (17, 18). Mutants which are absolutely unable to grow with sulfate as a sulfur source bind a few thousand sulfate ions per cell. This suggested that

a specific part of the transport system, the binding of sulfate, could be studied separately from the entire process. First it was shown that disrupted bacteria retained the binding ability. Then the binding component was isolated.

A new type of assay is required to identify the sulfate-binding activity of cell-free preparations (text-fig. 4). The basis of the assay is that a radioactive sulfate solution is equilibrated with the anion exchange resin Dowex-1. A small fraction of the total sulfate remains in solution. If a substance capable of binding sulfate is now added, more sulfate is released from the resin and brought into solution in a bound form. Thus, an increase in the radioactivity of the supernatant fluid is proportional to the amount of binding material, providing an assay for the amount of Binder.



TEXT-FIGURE 4.—Principle of the binding assay.

Purification was greatly facilitated by derepression, whereupon Binder reached 1% of the total protein. Osmotic shock released substantially all of the Binder. In the supernatant solution of shocked cells 20% of the total protein was Binder. Standard enzyme-isolation procedures achieved isolation of Binder, which was homogeneous by the conventional tests of protein biochemistry.

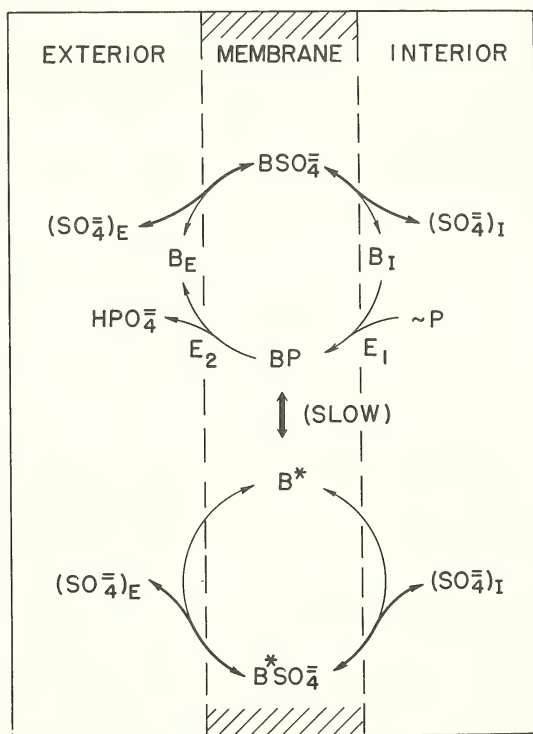
Binder turned out to be a protein of rather typical amino acid composition, with a molecular weight of about 32,000. It bound one sulfate per unit molecular weight, and the dissociation constant was very strong (about 10^{-8} M). The degree of dissociation depended upon ionic strength in such a way as to suggest that the binding was purely ionic (18).

A variety of tests indicates a close relation between binding and active transport (17). Both are repressible, both are absent in certain

mutants, and both are lost by bacteria which have been converted into spheroplasts or subjected to osmotic shock, which releases surface components. It seems very likely, then, that this sulfate Binder is indeed a long-postulated carrier (1, 4) in active transport.

A MODEL FOR SULFATE TRANSPORT AND ITS INHIBITION

These observations restrict the number of models that could explain sulfate transport. Experimental observations have shown the presence of Binder outside the membrane, no energy requirement for binding, absence of either active or passive transport without an energy supply, and mutants that possess binder but cannot transport. A highly speculative model consistent with these and other observations is given in text-figure 5.



TEXT-FIGURE 5.—A model for sulfate transport and its inhibition.

The upper cycle performs active transport. Neither Binder (B_E) nor free sulfate $(SO_4^{=})_E$ can penetrate the membrane, but combined Binder-sulfate ($BSO_4^{=}$) diffuses through readily. The latter can dissociate

and release sulfate (SO_4^-)₁ and free binder (B_1) inside the cell. Binder cannot return until an enzyme (E_1) has reacted it with a high-energy donor ($\sim\text{P}$), either ATP or possibly phosphoenol-pyruvate, as suggested by recent work on a protein involved in sugar transport (19). The phosphorylated Binder (BP) returns to the exterior of the membrane and is dephosphorylated there by a second enzyme (E_2). It can then transport again.

The lower part of the text-figure provides a mechanism for inhibition. A slow reaction is postulated to convert BP (which requires sulfate transport and energy for its formation) to a modified Binder (B^*), which penetrates the membrane freely with or without bound sulfate. "Facilitated diffusion" would gradually replace active transport and cause a net outflow of sulfate. Expressed mathematically, an equation like one derived earlier (14) is obtained. It shows an overshoot.

The problem now is to obtain biochemical evidence to learn if models of this type are correct.

RESUMEN

Los sistemas de transporte activos, por estar colocados al principio de los caminos metabólicos y a causa de los requerimientos de energía tanto para su formación como para su actividad, parecen ser puntos lógicos donde operen los mecanismos de control metabólico. Son muy pocos los casos de sistemas de transporte y sus mecanismos de regulación que se han analizado con detalle. Se conocen bien, en bacterias, los sistemas de transporte activo inducibles, aun cuando la importancia fisiológica de esta regulación no se haya aclarado por completo, lo que se ha hecho aun más complejo al comprender que existen muchos sistemas de transporte activos en una diversidad de ejemplos. La base genética para la regulación de estos sistemas parece ser idéntica y, de hecho, coincide cuando menos en un caso, con la regulación de la síntesis de proteínas.

La regulación por inhibición por producto final de la actividad de los sistemas de transporte ha sido objeto de exploraciones aun más discretas. Esto puede atribuirse en parte a nuestra incapacidad para manipular los sistemas, debido a la necesidad de contar con células intactas para estudiarlas. Se ha descrito un caso en el que el transporte de glucosa parece estar regulado por la acumulación de un producto metabólico de alta energía. En otro caso, el acto del transporte del sulfato parece estar regulado por algún intermediario de sulfato de alta energía. Se han hecho progreso en este último caso, debido al aislamiento de una parte del sistema que bien pudiera ser el transportador, a menudo postulado como el responsable del transporte, a través de membranas, de moléculas pequeña que sin él no atravesarían a las membranas. Se presenta un esquema para el transporte y la inhibición por retroalimentación de sulfato compatible con los datos conocidos.

Sin duda alguna, los modos de regulación de los sistema de transporte activo porporcionarán un campo amplio para la investigación.

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Arginine-Pyrimidine Pathways in Microorganisms^{1,2,3,4}

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SUMMARY

This paper discusses 1) the probable origin of branched biosynthetic pathways and 2) the evolution of regulatory systems adequate to deal with such paths. The biosynthesis of carbamyl phosphate (CP), a precursor common to arginine and pyrimidines, is used as an illustration. *Neurospora* and other fungi have developed two distinct enzymes for the synthesis of CP; one is subject to regulation by arginine and the other by pyrimidines. Furthermore, *Neurospora* has two separate CP pools, one for each path. This channeling is

labile under certain circumstances. Its nature is discussed. *Bacillus subtilis* has only one CP synthetase, in addition to a catabolic CP kinase. This is shown by the isolation of a CP synthetase-less mutant that requires both arginine and pyrimidines for growth, and by studies on the regulation of this enzyme. It displays concerted repression and cumulative feedback inhibition by arginine and pyrimidines. This situation is compared with that found in other bacteria.—Nat Cancer Inst Monogr 27: 259–271, 1967.

IN THE four decades that have elapsed since Kluver and Donker (1) published their paper on “the unity in biochemistry,” comparative biochemistry has evolved from the stage of brilliant generalizations to

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² See Discussion of this paper conducted by Dr. R. L. Metzenberg, p. 297.

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⁷ While this manuscript was in preparation we had the privilege of seeing the draft of a paper by Rowland H. Davis [in press in *Organizational Biosynthesis* (H. J. Vogel, J. O. Lampen, and V. Bryson, eds.). Academic Press Inc.] which disclosed results very similar to those reported here. We are grateful to Dr. Davis for this communication, as well as for the intellectual stimulation received from him throughout the years. We also wish to acknowledge the able technical assistance of Elba T. de Gros and Mateo L. Cosmelli.

that of making predictions, formulating laws, and even being able to stumble gracefully upon the exceptions. Such laws, of course, fall back upon the principles of molecular biology, genetics, and evolution, and it is in terms of the strategy of the latter that causal answers are couched. Thus, two decades ago Horowitz (2) published an important paper in which he suggested that biochemical pathways evolved starting with the end product of the chain and working backward toward the beginning by the stepwise acquisition of the necessary enzymes. Each step, Horowitz felt, was prompted by the diminishing supply of precursors in the external environment. This very plausible mode of evolution would favor the development of branched pathways, since a chain could easily solve its supply problem by feeding on an intermediate of another chain. Some complications would ensue, however, with the development of regulatory systems.

Only in the last decade have biochemists become aware of the importance of regulatory circuits in metabolism. Numerous circuits have been identified, and their design is much the same as would be expected for maximal efficiency. Yet, the type of circuit useful for linear pathways would clutter up a branched one. How did evolution cope with this problem? The pioneer work of Stadtman *et al.* (3) uncovered one solution. They studied in *Escherichia coli* the biosynthesis of aspartyl phosphate, a step prior to the point where the lysine and threonine paths branch, and found two different aspartokinases to catalyze this step. One enzyme is subject to threonine regulation and the other to lysine regulation. One might consider these chains as sharing a step in parallel, and not as true branching; however, in the light of the evolutionary considerations presented earlier, it appears likely that the parallel enzymes evolved only after true branching had been established.

Evolution is essentially opportunistic and as such tends to provide different solutions for the same problem. It is therefore not surprising that the multiple-enzyme solution is only one of several (4). Other solutions involve the joint action of the various end products on a single enzyme located prior to the branching point. Thus, feedback inhibition may be cumulative, cooperative, or concerted (multivalent), depending upon whether the various effectors act on the enzyme additively, synergistically, or only in the presence of all others. Similarly, instances of cumulative and concerted repression are known.

Arginine and pyrimidines are synthesized in microorganisms via a common precursor: carbamyl phosphate (CP). Their biosynthesis provides a typical example of a branched pathway, and may serve to illustrate factually the generalities presented above.

DUAL ENZYMES FOR THE SYNTHESIS OF CARBAMYL PHOSPHATE IN *NEUROSPORA*

Pathways

Davis (5) has demonstrated in *Neurospora* that the arginine-requiring mutants *arg2* and *arg3* are deficient in a glutamine-dependent carbamyl phosphate synthetase activity (GCPS).⁸ He suggests that the enzyme is made up of two different polypeptide subunits, controlled respectively by *arg2* and *arg3*. The next step in the synthesis of arginine is the formation of citrulline from carbamyl phosphate and ornithine. This step is catalyzed by ornithine transcarbamylase (OTC). The structural gene for OTC is *arg12* (8).

Carbamyl phosphate is also a precursor for the synthesis of pyrimidines in *Neurospora*, as shown by the fact that aspartic transcarbamylase (ATC), an enzyme catalyzing the synthesis of carbamyl aspartate from carbamyl phosphate, is indispensable for pyrimidine synthesis (9). The structural gene for ATC is *pyr3N*⁹ (10, 11). How then, can a block in carbamyl phosphate synthesis create a nutritional requirement for arginine, but not for pyrimidines?

Let us assume, as has been done for some time (12, 13), that CP is synthesized in *Neurospora* by dual enzymes, one for each pathway, much in the manner of bacterial aspartokinase. The facts just mentioned are then easily understood on two additional assumptions: that the two CP synthetases feed into separate CP pools, and that the assay used for GCPS only detects the enzyme of the arginine path (GCPSa). A special effort to detect GCPS activity in GCPSa-less mutants is thus in order. We have very recently succeeded in detecting such an activity in *arg3*, after grinding in liquid nitrogen and passing the extract through Sephadex. This activity is highly labile, but quite reproducible under carefully controlled conditions. It is not detectable in extracts of *arg3 pyr3M*, a double mutant requiring arginine and pyrimidines for growth. Therefore, this labile CP synthetase is a different enzyme from GCPSa and is under the control of the gene *pyr3M*. It will be called GCPSp because it uses glutamine as a nitrogen donor (table 1). Ammonia can substitute for glutamine, but does not fare so well. The assay system used is based on the incorporation of ¹⁴C-bicarbonate into the acid-stable fraction, and thus requires linking CP production with another reaction stabilizing the carbamyl moiety. This is conveniently accomplished by addition of either aspartate or ornithine to

⁸ It is customary to distinguish between carbamyl phosphate synthetases, which utilize two moles of ATP per mole of CP, and carbamyl phosphokinases (CPK), which utilize only one mole. The former are anabolic and the latter catabolic. The CPK enzymes use ammonia as a source of nitrogen, and the synthetases (GCPS) may use either ammonia (6) or glutamine (7). Thus, it is often possible to determine whether one is dealing with the kinase or the synthetase by considering its metabolic role and nitrogen donor. Our choice of terminology will be guided accordingly.

⁹ Other authors usually identify this gene as *pyr3d*, which is the designation of one of its alleles.

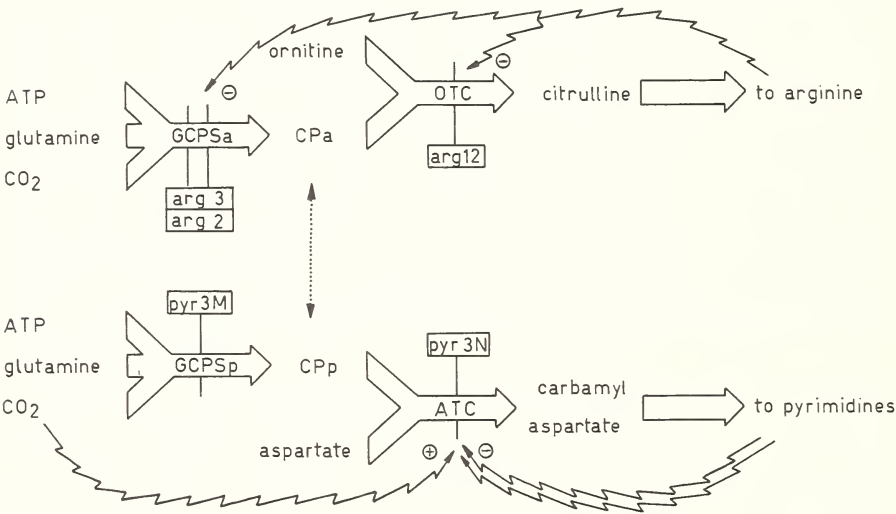
the incubation mixture, because there is excess ATC and OTC in the extracts. Specific activities of GCPSp in crude extracts of *arg3* are of the order 0.1 μ moles/hr/mg protein. (Text-fig. 1 summarizes the relationships discussed above.)

TABLE 1.—Requirements for the GCPSp reaction

Incubation mixture*	Relative activity†
Omissions	
Aspartate.....	0.36
Glutamine.....	0.09
ATP.....	0.02
Substitutions	
Ornithine for aspartate.....	0.63
Ammonia for glutamine.....	0.58

*In 0.5 ml (in μ moles): Tris-acetate, pH 8.4, 100; $MgSO_4$, 10; ^{14}C - $KHCO_3$, 10 (6 μc); aspartate, 7.5; glutamine, 6.7; ATP, 10; and extract. We prepared the extract by grinding young cultures of *arg3* under liquid nitrogen in a mortar, taking it up in a few ml of 0.02 M Tris-acetate/g wet weight, homogenizing gently, centrifuging for 5 min. at 20,000 $\times g$, and passing an aliquot of the supernatant through G-25 Sephadex (all steps performed in the cold). The reaction mixture was incubated for 20 minutes at 37 C. Then 0.3 ml of 1 N HCl was added and radioactivity was measured in a gas-flow counter.

† Values are expressed relative to incorporation in complete system, and are the average of 4 experiments.

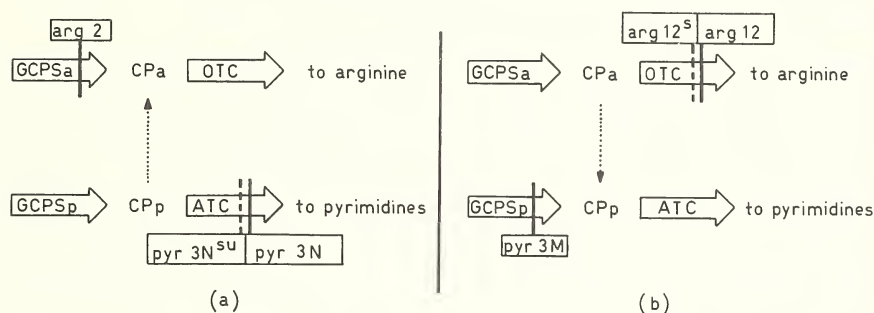


TEXT-FIGURE 1.—The biosynthesis of arginine and pyrimidines in *Neurospora*. Wavy arrow with \ominus indicates repression (14), with \oplus indicates induction (15). Double wavy arrow indicates repression and feedback inhibition (16). The regulatory regime of GCPSp has not been studied, but it is likely to involve repression by pyrimidines and induction by CO_2 (15). For other explanations, see text.

De-Channeling

The two pools of CP illustrated in text-figure 1 are necessary to explain the arginine requirement of *arg2* and *arg3*, and the pyrimidine

requirement of *pyr3M*. Another way to describe this is to say that the two pathways are channeled—or that at least one of them is, since effective channeling in one will suffice to isolate the pools. However, under special circumstances, which we shall review directly, *arg3*, *arg2*, and *pyr3M* can dispense with their nutritional requirements. By a reversal of the former argument, this shows that the two CP pools can then feed into each other. Such “de-channeling” (indicated in text-fig. 1 by the *dotted arrow*) is observed, for instance, in the double mutants (text-fig. 2), where a biochemical block further up the chain acts as a suppressor of the GCPS mutant in the opposite path.



TEXT-FIGURE 2.—Two types of double mutants in which the requirements imposed by GCPS mutations are suppressed by a second mutation (13, 14, 17, 18). *Solid lines* across the arrows indicate tight blocks; *dashed lines* indicate partial blocks and the *dotted arrows* indicate de-channeling. Double mutant (a) is a pyrimidine auxotroph when the *pyr3N* block is tight, but prototrophic for the partial (subthreshold) blocks at this locus (*pyr3N^{su}*). Double mutant (b) can likewise be either arginine-dependent or prototrophic.

Let us first consider the case of *arg2*, *pyr3N^{su}*. The suppression of its arginine requirement tends to vanish if such a mutant is cultured in the presence of uridine and in the absence of carbon dioxide (table 2, first column). The same kind of effect is observed in the residual growth of *arg2* in arginine-free medium (table 2, middle column), indicating that in *arg2 pyr3N⁺* strains there is also some degree of de-channeling. The effects of carbon dioxide and uridine are easily understood if we assume that uridine inhibits GCPS_p and that carbon dioxide stimulates this step. The first assumption is only too plausible if the possession of two GCPS is to have any selective value for *Neurospora*. The second could be either a mass action effect of carbon dioxide or the result of enzyme induction by the first substrate (15).

In summary, de-channeling seems to require a combination of an adequate flow along GCPS_p and a large ratio of GCPS_p to ATC. It is

TABLE 2.—Growth of *arg2* in the absence of arginine: stimulation by *pyr3N^{su}* and carbon dioxide, and inhibition by uridine

Uridine (mg/1)	Carbon dioxide*	Relative growth without arginine†		
		<i>arg2 pyr3N^{su}</i>	<i>arg2 pyr3N⁺</i>	<i>arg2⁺ pyr3N⁺</i>
0	Atmospheric	0.89	0.18	0.89
100	Atmospheric	0.72	0.09	1.02
0	0	0.72	0	0.73
100	0	0.30	0	0.74

*Deprivation of CO₂ is obtained by culturing in plates placed in desiccators over KOH solution.

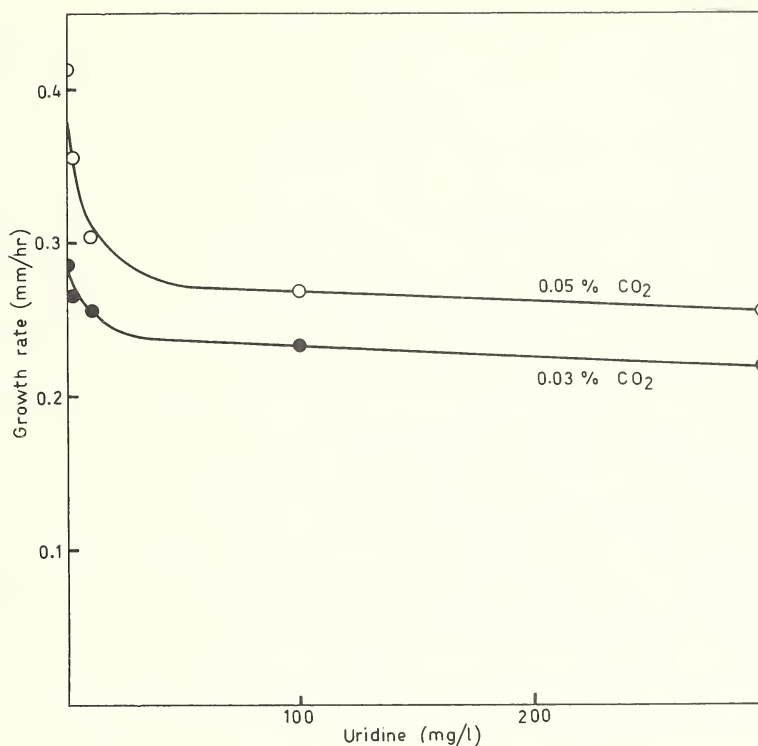
† Growth measured as in (15) and expressed relative to growth with arginine supplement. All mutants are coisogenic, since *arg2 pyr3N^{su}* and *arg2⁺ pyr3N⁺* were derived from *arg2 pyr3N⁺* by single-step mutation. Values for *arg2 pyr3N^{su}* and *arg2⁺ pyr3N⁺* are averages for 9 and 4 mutants, respectively.

difficult to separate these two factors because low ATC tends to reduce the pool of pyrimidines, and thus to stimulate flow along GCPSp. However, since excess uridine eliminates some, but not all, of the residual growth in *arg2* (text-fig. 3) and in *arg2 pyr3N^{su}*, it appears that both factors are of consequence. Furthermore, among over 100 mutants selected as suppressors of *arg2*, all affected ATC (17). This indicates that an increment in the GCPSp/ATC ratio is essential for the recovery of suppressors. A twofold reduction in ATC specific activity will suffice for full suppression (10). Jobbágy (11) has been studying the properties of the ATC in 5 different *pyr3N^{su}* mutants. In each case, the K_m for aspartate of ATC is larger than that of the wild type. The extreme case is mutant K0492-33, which has a K_m of 0.2 as compared to the normal value of 6×10^{-3} . On the other hand, its K_m for CP appears normal, and the pH optimum is shifted from 9.5–8.5. The reasons for this selection of mutants altered in the K_m for aspartate are not apparent.

The double mutant *pyr3M arg12^s* [text-fig. 2(b)] behaves in a fashion essentially symmetrical to *arg2 pyr3N^{su}*, but the former is far more sensitive to arginine than the latter is to uridine (19). Analogous behavior is exhibited by *pyr3M* alone: Its residual growth on pyrimidine-free media is stimulated by carbon dioxide and inhibitors of OTC, and markedly inhibited by arginine (20–22). It appears likely that in this situation the prime factor in de-channeling is the lowering of the end-product pool.

Channeling

We have just seen that channeling can be easily disturbed, but this does not enlighten us very much about the nature of channeling; at most it eliminates some alternatives. Theoretically, we may consider models implying either temporal or spatial separation of pools. The



TEXT-FIGURE 3.—Partial inhibition of the residual growth of *arg2* by uridine. Growth measured as in (15). In the presence of arginine, rate was 1 mm/hour, regardless of uridine or carbon dioxide concentration.

first possibility is only speculative (10), and assumes that the oscillatory behavior of the regulatory processes (23) is such that the two paths are out of phase with each other. The second alternative requires either 1) compartmentalization of cell constituents, 2) specific association of protein molecules, or 3) multifunctional enzymes. Evidence in support of alternative 3 has been presented on the basis that the *pyr3N* and *pyr3M* loci map as contiguous genes, with some overlap, and that mutations affecting both *M* and *N* may revert to partial restoration of either the *M* or the *N* function (24–26). This is very suggestive evidence, but not compelling in view of the phenomenon of polarity (27). What one would really like to have is an *in vitro* system exhibiting at least some measure of channeling. Yet table 1 shows that both ornithine and aspartate were effective as acceptors for the CP synthesized by GCPSp *in vitro*. It is true that the latter was more effective than the former, but CP is known (14) to have a higher affinity for ATC than for OTC. On the other hand, under the condi-

tions of our assays OTC is eightfold more active than ATC. To have a basis for comparison, the experience was repeated, this time using a *pyr3M* mutant as an enzyme source. Once again, ornithine was close to 60% as effective as aspartate as a CP acceptor. Thus, there are no indications *in vitro* of preferential coupling between GCPSa (*pyr3M* extracts) and OTC or between GCPSp (*arg3* extracts) and ATC. We conclude that channeling does not survive grinding in liquid nitrogen. This argues against alternative 3 above. However, as has been pointed out to us by Rowland H. Davis, channeling along the postulated MN multifunctional enzyme may require the presence of the substrates for the second step. This condition was not fulfilled by the incubation mixtures used. For a more extensive discussion, see R. H. Davis in *Organizational Biosynthesis*, H. J. Vogel, J. O. Lampen, and V. Bryson, eds. (Academic Press Inc., in press).

In summary, *Neurospora* has chosen enzymatic multiplicity (*i.e.*, two GCPS enzymes) as a solution for its regulatory problems in the arginine-pyrimidine pathway. In addition, a partial segregation of the CP pools for the two pathways is observed. The multiple-enzyme solution has also been adopted by two other fungi: *Saccharomyces cerevisiae* (28) and *Coprinus radiatus* (29). The CP pools appear to be segregated in *Coprinus*, but not in *Saccharomyces*. In both cases, as in *Neurospora*, the genes specifying GCPSp and ATC may mutate simultaneously (*i.e.*, they are part of the same unit of transcription).

ONLY ONE CARBAMYL PHOSPHATE SYNTHETASE IN *BACILLUS SUBTILIS*

In contrast to the fungi discussed above, in the few cases in which the synthesis of carbamyl phosphate has been studied in bacteria, the evolutionary solution seems to be of the single enzyme type. This is shown in *Escherichia coli* and in *Salmonella typhimurium* by the fact that single-step mutants creating double auxotrophy for arginine and pyrimidines (30, 31) lack GCPS activity (32); Irene Huberman, unpublished results. We will now report on similar results obtained with *B. subtilis*.

In a search for arginine-pyrimidine double auxotrophs, after ultraviolet irradiation at the 1% level and screening by the penicillin method (33), two such mutants (*cap1* and *cap2*) were isolated among 3 arginine-dependent and 13 pyrimidine-dependent mutants.

The next step was to investigate CP synthetase activity in wild and mutant extracts. The matter was not straightforward because other enzymes interfered with the synthetase assay. To make a long story short, let us first dispose of the interferences in a perfunctory fashion. Interference number one was sensitive to acetate and was probably

caused by acetylphosphokinase (34). It had to be eliminated when ammonia was used as source of nitrogen, and this was conveniently done by addition of acetate. A more insidious problem derived from the presence of an ammonium-dependent CPK (35). Fortunately, this activity could be separated from the synthetase by ammonium sulfate fractionation. It was thus shown that while CPK uses ammonia as source of nitrogen, the synthetase is glutamine-dependent (GCPS). The pH optima for CPK and GCPS are 8.5 and 7.5, respectively. The GCPS could be assayed specifically in crude dialyzed extracts at pH 7.5 by use of glutamine as nitrogen donor. The addition of glutathione helped to stabilize this labile enzyme. The enzyme requires ATP and Mg^{++} for activity.

Very little GCPS activity (0.1 μ mole/hr/mg protein, or less) was detected in extracts of *cap1* grown on limiting arginine and uracil (10 and 2 mg/liter, respectively, methods as in table 3). This fact, together with the studies on regulation to be presented below, confirms that *B. subtilis* uses a single enzyme for the synthesis of CP.

The next question we asked ourselves was about the manner of regulation of this enzyme. Repression was investigated by comparison of specific activities of GCPS from wild-type cells cultured in various media. The results (table 3) indicate that there is no clear effect (or perhaps some induction) when either end-product is added separately, but there is marked repression by the simultaneous presence of arginine and uracil in the medium. Repression is therefore concerted.

TABLE 3.—Concerted repression of *Bacillus subtilis* GCPS by arginine and uracil

Supplements added to culture media*	Relative specific activities of GCPS†			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Arginine.....	1.1	—	2.4	0.4
Uracil.....	1.4	1.6	3.8	1.2
Arginine plus uracil.....	0.06	0.03	0.15	0.09

*Added to the basic medium [minimal (36) with 0.1% casaminoacids instead of yeast extract and 0.5% glucose] as 200 mg/liter arginine and 100 mg/liter uracil. Grown for 20 hours at 37 C in a 2 liter volume with 100 ml inocula pregrown overnight in the same medium.

† Values relative to contemporary cultures from unsupplemented medium. Specific activities in these cultures ranged from 0.32–4.4 μ mole/hr/mg/protein in the 4 experiments performed at different times and with some changes in methodology. Incubation mixture (μ moles in 1–2 ml): Tris pH 7.5, 100; ^{14}C -Na bicarbonate, 20 ($2-4 \times 10^5$ cpm); glutamine, 10; ATP, 20; $MgCl_2$, 20; and bacterial extract (2–3 mg protein). Cells were broken by homogenizing with glass beads; supernatant was dialyzed against Tris buffer with glutathione or mercaptoethanol-EDTA. Assayed for ^{14}C incorporation after conversion of CP to urea (7).

We then investigated the inhibitory effect of some metabolites added to the incubation mixture at 10^{-2} M concentration. We found inhibition by arginine and by several pyrimidines (table 4). Since UTP is the most active pyrimidine derivative, it was singled out for further study. Its maximum effect is already achieved at 10^{-3} M.

TABLE 4.—Inhibition of GCPS

Additions to incubation mixture (0.01 M)	Relative activity*	
	Expt. 1	Expt. 2
Uridine.....	0.46	0.59
UMP.....	1.06	0.98
UTP.....	0.25	0.37
CMP.....	0.81	0.56
CTP.....	0.80	0.77
Citrulline.....	1.00	0.90
Arginine.....	0.66	0.64

*Values relative to activity with no addition. Cultures from uracil-supplemented medium. Methods as in table 3.

A good quantitation of feedback effects is hampered by a somewhat variable behavior of the enzyme toward its inhibitors. Furthermore, our work was performed with crude preparations. Given such reservations, it is apparent (table 5) that inhibition by arginine plus UTP is cumulative or, in other words, that the sum of the two inhibits as expected for two compounds acting independently.

Our studies on the regulation of GCPS also have a bearing on the problem of whether *B. subtilis* has one or two GCPS enzymes. In the first place, the concerted pattern of repression observed is only expected if a single enzyme was involved. Second, if we still wish to consider the possibility that two enzymes exist and that each is repressed in turn by arginine and uracil, then we would expect the pattern of feedback inhibition to vary according to whether the preparation came from an arginine or uracil culture. But this is not the case (table 5).

TABLE 5.—Pattern of feedback inhibition by arginine and UTP on GCPS from cultures in different media

Supplements added to culture media	Relative activity in the presence of inhibitor*		
	Arginine (0.01 M)	UTP (0.001 M)	Arginine plus UTP (0.01 M: 0.001 M)
None.....	1.12 0.78	0.08 0.02	0.12 0.00
Arginine.....	1.00 0.35 0.98	0.28 0.14 0.05	— 0.08 0.17
Uracil.....	0.81 0.60 0.63	0.00 0.11 0.14	0.01 0.00 0.00

*Values relative to assay of same extract in the absence of inhibitor. Growth media and assay methods as in table 3. Each line is one experiment.

In summary, *B. subtilis* has a single enzyme for CP synthesis which is subject to concerted repression and cumulative feedback inhibition by arginine and pyrimidines. The situation is to be compared with that found in *E. coli*, where a single GCPS responds to cumulative repression by these same end-products and to feedback inhibition only by pyrimidines (37).

We began discussing the strategy of evolution. Let us now conclude by making one minor point, one that is possibly relevant to this grand strategy. *B. subtilis* possesses one of the few species of ATC which is not inhibited by pyrimidines (38). In such a situation one would expect to find that GCPS would be inhibited by pyrimidines, and that this inhibition would not be conditioned by the presence of arginine (*i.e.*, that it would not be concerted but cumulative). It was gratifying that our findings agreed with these expectations.

RESUMEN

En este trabajo se analiza el origen de las cadenas biosintéticas ramificadas y la evolución de los sistemas de regulación que las controlan. La biosíntesis del carbamil fosfato, precursor a la vez de la arginina y de las pirimidinas, se utiliza como ejemplo.

La *Neurospora* y otros hongos han desarrollado dos enzimas diferentes para la síntesis del carbamil fosfato: la una regulada por el nivel de arginina; la otra, por el nivel de pirimidinas. Además, la *Neurospora* tiene dos reservorios diferentes para el carbamil fosfato: uno utilizado en la vía de la arginina y el otro en la vía de las pirimidinas. Esta canalización del carbamil fosfato hacia una y otra vía puede desaparecer bajo circunstancias especiales.

En *Bacillus subtilis*, en cambio, hay una sola sintetasa de carbamil fosfato (aparte de una carbamil fosfoquinasa cuya función fisiológica es degradativa). Esto se demuestra por el aislamiento de un mutante que requiere arginina y pirimidinas para crecer por carecer de dicha sintetasa. Estudios sobre el régimen de regulación de esta enzima confirman la conclusión anterior y muestran que la misma está sujeta a represión concertada y retroinhibición cumulativa por arginina y pirimidinas. Este régimen de regulación se compara con el que prevalece en otras bacterias.

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Catabolism of L-Arginine in *Neurospora Crassa*^{1,2}

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SUMMARY

The catabolism of endogenous bio-synthesized arginine and exogenous arginine added to the culture medium has been studied in the fungi *Neurospora crassa*. In this mold, the basal pool of endogenous L-arginine is not available for catabolism. When the endogenous pool of this amino acid is increased by the addition of L-citrulline to the culture medium, the endogenous L-arginine becomes available for catabolism. A higher pool of endogenous L-arginine and a fully induced arginase, ornithine transaminase, and urease are not sufficient conditions to assure an efficient endogenous catabolism of this amino acid; also ammonium

nitrate must be present in the culture medium. When this compound is absent, exogenous arginine versus endogenous is catabolized. A mutant of *N. crassa* is unable to catabolize exogenous arginine in the presence of ammonium nitrate. Experiments with ¹⁴C-guanido L-arginine indicate that in *N. crassa*, under conditions of arginine excess, catabolic ornithine from the arginase reaction is not available for arginine biosynthesis—therefore, establishing the inoperation of the ornithine cycle in this micro-organism.—Nat Cancer Inst Monogr 27: 273–282, 1967.

ALL THE enzymes that participate in the urea cycle are present in the mold *Neurospora crassa*, but in contrast to ureotelic organisms (1–4), wild-type *N. crassa* does not require L-arginine for optimal growth (5), an arginine pool is easily detectable, ammonia is not a toxic compound, and a glutamine-dependent carbamyl-phosphate synthetase (6) [instead of an ammonia-dependent carbamyl-phosphate synthetase (4)] and urease (7) are present in cell-free extracts. Also, we have recently found that the fungal arginase is a protein very

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² See Discussion of this paper conducted by Dr. R. L. Metzenberg, p. 297.

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similar to an arginase found in the liver of uricotelic animals but different from that present in the liver of ureotelic animals (8, 9).

These facts—together with the demonstration in *Neurospora* that the basal endogenous pool of L-arginine is not, under certain conditions, available for catabolism (10)—suggest that the role of ureotelic arginase is the hydrolysis of endogenous arginine, supplying L-ornithine for further ammonia fixation in the urea cycle; whereas the function of the fungal arginase is the hydrolysis of exogenous L-arginine, providing L-ornithine and urea as a source of L-glutamic acid nitrogen, L-proline, and ammonia. Thus, the endogenously synthesized arginine in this fungus is mainly restricted to protein biosynthesis.

To test this hypothesis and to examine further the metabolic fate of exogenous and endogenous biosynthesized L-arginine, a study has been undertaken to analyze the catabolism of exogenous and endogenous L-arginine in *N. crassa*, wild-type 74A, the mutant strain auxotrophic for L-proline, *arg-8*, and in the new mutant, *INO*.

The catabolism of exogenous L-arginine has been previously studied in *N. crassa* 74A and in the mutant *arg-8* by growing these strains in L-arginine as a nitrogen and L-proline source, respectively (11). The catabolism of endogenous arginine has been studied similarly by supplying a precursor of this amino acid, L-citrulline, to the medium.

The rationale of studying the catabolism of L-arginine in this way has been discussed already (11), and is as follows. First, arginase is the only activity detectable in cell-free extracts of *N. crassa* that utilizes L-arginine as substrate. Through the action of this enzyme, ornithine and urea are produced. By the action of a ornithine- δ -amino transaminase present in *N. crassa*, the former compound transaminates with α -keto glutaric acid and is converted to glutamic- γ -semialdehyde, which cyclizes nonenzymatically to Δ' -pyrroline-5-carboxylate, and is subsequently reduced to L-proline. Urease, also present in *N. crassa*, hydrolyzes urea to ammonia and CO₂ (see scheme in text-fig. 1). Second L-arginine is 1) a nitrogen source in strain 74A; 2) a substitute for L-proline in the mutant *arg-8*, which has a block between glutamic acid and glutamic- γ -semialdehyde; and 3) a source of glutamic acid in the mutant *am1*, a strain that lacks glutamic dehydrogenase. Since exogenous L-citrulline also substitutes for L-proline in *arg-8* but does not substitute for glutamic acid in the strain *am1*, the catabolism of endogenous arginine was assumed to be a necessary condition for growth when L-citrulline is used as a nitrogen source in 74A and as a proline source in *arg-8*.

Except where indicated, conditions for growth of the mold, the cell-free extraction, and the assay of enzymes and pools were as previously described (11).

In text-figure 1 we have summarized the results obtained when ammonium nitrate, L-arginine, and L-citrulline are used as nitrogen sources in strain 74A (11).

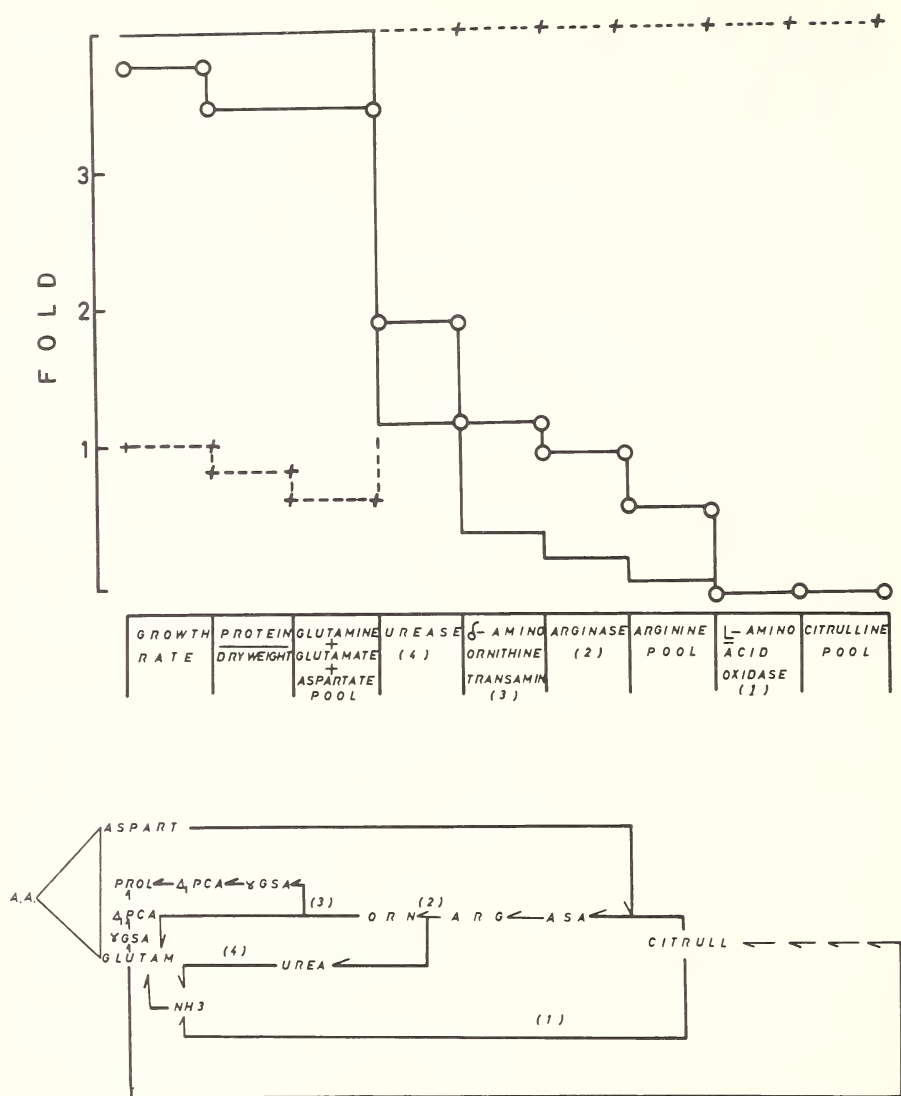
Judged by its growth rate, protein/dry weight ratio, and amino-nitrogen pool, exogenous L-arginine is almost as good a nitrogen source as ammonium nitrate. Compared to the values found in ammonium nitrate, growth of the mold in L-arginine as sole nitrogen source is accomplished with a relatively moderate increase of the arginine pool, arginase, ornithine transaminase, and urease activities.

Surprisingly, L-citrulline is not a good nitrogen source in *Neurospora* despite the remarkable increase in the citrulline and arginine pools and in the activities of L-amino acid oxidase, arginase, ornithine transaminase, and urease. The ammonium deficiency that occurs with L-citrulline as a nitrogen source is prevented when ammonium nitrate or L-arginine is also present, and reversed when these compounds are added to a culture previously grown in L-citrulline. The severity of the ammonium starvation depends on the type of inoculum used, being higher when the inoculum is unblended mycelium than when it is blended mycelium or conidia. The greater the severity of ammonium deficiency, the higher the citrulline and arginine pools and the activities of the enzymes mentioned above (11).

These experiments suggest that, in the absence of ammonium nitrate, exogenous arginine is preferentially catabolized by arginase over endogenous arginine. In the presence of ammonium nitrate, endogenous arginine is available for catabolism. This has been inferred from the growth of *arg-8* in the presence of L-citrulline and ammonium nitrate. Under this condition there is a moderate elevation of the citrulline and arginine pools and of the activities of arginase and ornithine transaminase. L-Lysine, a potent inhibitor of the arginase *in vitro*, inhibits the growth of *arg-8*, under this condition. The lack of growth of this mutant in minimal medium must be attributed to the unavailability of the basal endogenous arginine pool for catabolism by arginase. When ammonium nitrate is absent, the metabolic behavior of *arg-8* in the presence of L-citrulline is similar to that already reported for strain 74A (11).

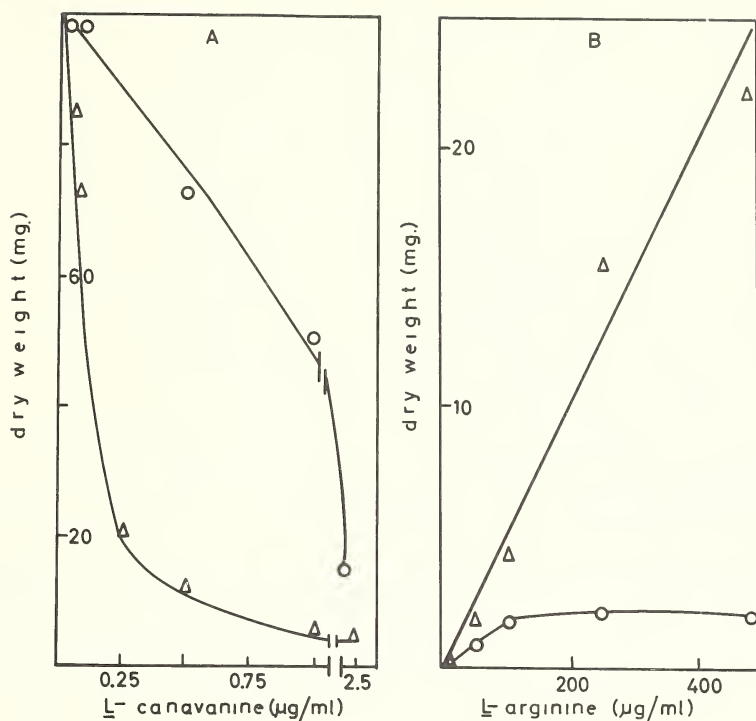
A mutant of *arg-8* has been found that is resistant to L-canavanine, and in the presence of ammonium nitrate does not grow in L-arginine. In text-figure 2A are shown the different sensitivities to L-canavanine of the growth of an inoculum of blended mycelium in proline, of the parental strain *arg-8*, and of the new mutant *INO*. Text-figure 2B shows that arginine reverses the inhibitory effect of L-canavanine on the growth of *arg-8*, but not on the growth of *INO*.

This is explained by the fact that *INO* does not grow in L-arginine in the presence of ammonium nitrate, as shown in text-figure 3A, where it may also be seen that this mutant grows in L-arginine in the



TEXT-FIGURE 1.—Growth, amino acid pool(s), and enzyme activities involved in α-amino-nitrogen formation when *Neurospora crassa* is grown in: 25 μmole/ml of ammonium nitrate (continuous line); 2000 μg/ml of L-arginine (line with circles); and 2000 μg/ml of L-citrulline (discontinuous line). Inoculum: unblended mycelium.

absence of ammonium nitrate. Text-figure 3B and 3C shows that the arginase is not induced nor the ornithine transcarbamylase repressed by L-arginine when ammonium nitrate is present in the growth medium of *INO*.

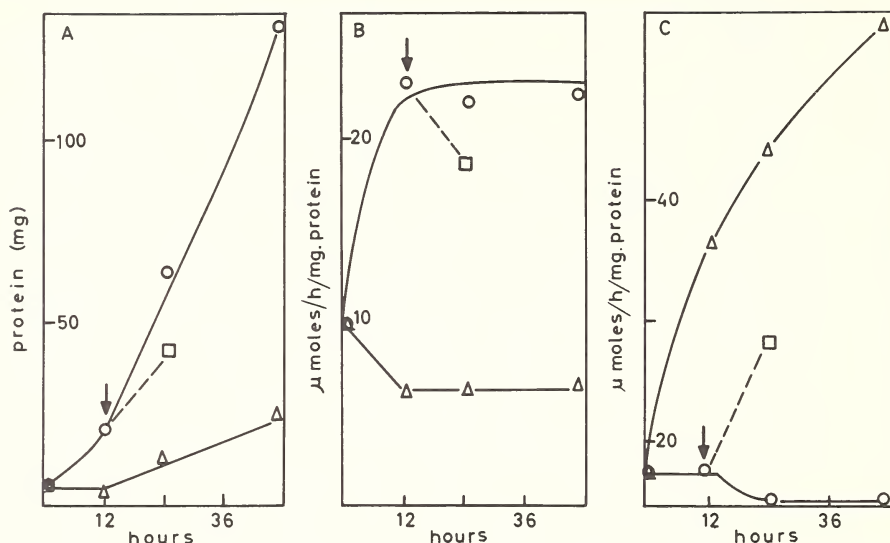


TEXT-FIGURE 2.—A: Effect of different concentrations of L-canavanine on 24-hour growth of *arg-8* (Δ) and *INO* (\circ) in the presence of 200 $\mu\text{g/ml}$ of L-proline in Vogel's minimal medium. 2B: Effect of different concentrations of L-arginine on the growth of *arg-8* (Δ) and *INO* (\circ) in the presence of 2.5 $\mu\text{g/ml}$ of L-canavanine Vogel's minimal medium. Inoculum: blended mycelium.

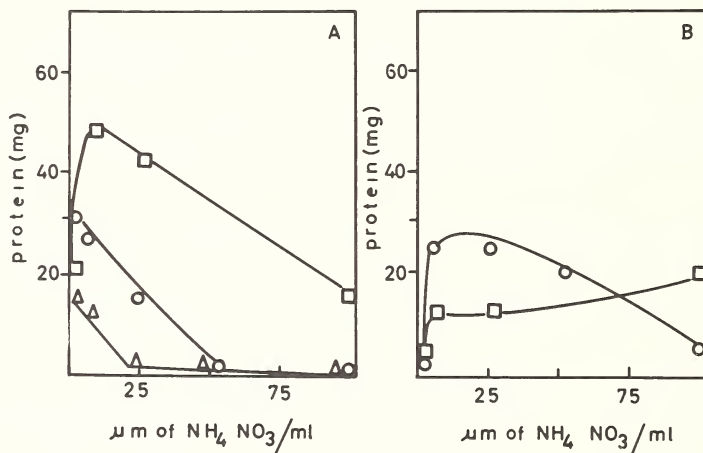
A logical conclusion is that, in the presence of ammonium nitrate, *INO* is not permeable to L-arginine, and, as a prediction, it is impermeable to L-canavanine. This is in accord with previous data which suggest that in *N. crassa* (12), as in other microorganisms (13), the same mechanism facilitates the entry of L-arginine and L-canavanine.

In text-figure 4 are shown effects of different concentrations of ammonium nitrate on the growth of *arg-8* and *INO* in L-arginine (A) and L-citrulline (B). In the absence of ammonium nitrate, the growth of these strains in L-arginine is very similar, but upon presentation of ammonium nitrate, the growth of *INO* is inhibited, whereas that of *arg-8* is stimulated. This stimulation disappears at higher ammonium concentrations.

In text-figure 5 is shown the amount of radioactivity in the evolved CO_2 and in the arginine pool, found in cultures of *arg-8* and *INO* grown in the presence of ^{14}C -guanido L-arginine and different concentrations of ammonium nitrate.

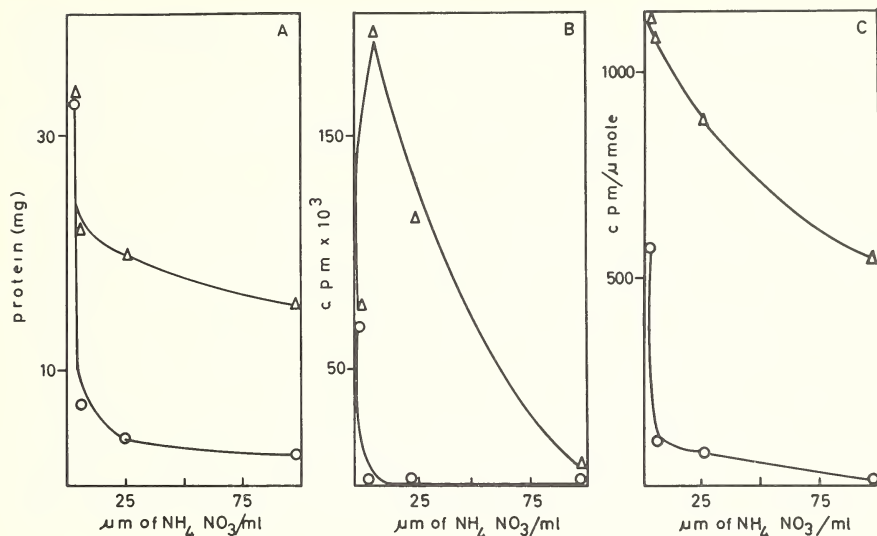


TEXT-FIGURE 3.—Growth of *INO* in the presence of 800 $\mu\text{g/ml}$ of L-arginine with (Δ) and without (\circ) 100 $\mu\text{mole/ml}$ of ammonium nitrate. A: Growth. B: Specific activity of arginase. C: Specific activity of ornithine transcarbamylase. Arrow indicates the addition of 100 $\mu\text{mole/ml}$ of ammonium nitrate to a culture previously grown in the absence of this compound. Inoculum: blended mycelium.



TEXT-FIGURE 4.—Effect of different concentrations of ammonium nitrate on 24-hour growth of *arg-8* (\square) and *INO* (\circ) in the presence of 800 $\mu\text{g/ml}$ of L-arginine (A) or 800 $\mu\text{g/ml}$ of L-citrulline (B). Growth of *INO* in 200 $\mu\text{g/ml}$ of L-arginine (Δ).

In the mutant *INO*, the decrease in growth (text-fig. 5A) when ammonium nitrate is present parallels the decrease in radioactivity in both the CO_2 (text-fig. 5B) and arginine pools (text-fig. 5C). A very



TEXT-FIGURE 5.—Effect of different concentrations of ammonium nitrate on 24-hour growth of *arg-8* (Δ) and *INO* (○) in the presence of 800 μg/ml of ¹⁴C-guanido L-arginine with a specific radioactivity of 1060 cpm/μmole. A: Growth. B: Radioactivity trapped in barium carbonate. C: Specific radioactivity of the arginine pool. Inoculum: blended mycelium.

different effect is seen in *arg-8*, where only at higher concentrations of ammonium nitrate no radioactivity was found in CO₂. It is interesting that in the absence of ammonium nitrate the arginine pool in *arg-8* was completely labeled, but in *INO* only 50% was labeled. The higher sensitivity to ammonium nitrate shown by *INO* in the latter experiment may be due to the fact that oxygen was bubbled into the cultures in the collection of the CO₂.

Different effects of ammonium nitrate are observed on growths of *arg-8* and *INO* in L-citrulline. The growth of *INO* is stimulated by intermediate concentrations and inhibited almost completely by higher concentrations, whereas that of *arg-8* is stimulated by all concentrations (text-fig. 4B).

Though it is premature to designate conclusively the specific phenotypic lesion of the new mutation *INO*, we propose that this mutant lacks a permease for L-arginine with which to transport this amino acid to a biosynthetic pool.

The importance of the new mutant *INO* is that, in the presence of ammonium nitrate, it is able to catabolize only endogenous sources of arginine.

The experiments reported up to this point support the assumption that *Neurospora* distinguishes endogenous and exogenous arginine.

The metabolic fate of arginine depends on this distinction, which seems to be regulated by ammonium nitrate. From the regulatory point of view, ammonium nitrate must be related to amino acids as glucose is related to carbohydrates as a carbon source. The regulation of the entry of amino acids into the cell can be a key metabolic point in deciding its metabolic fate.

The results presented in this work can be explained by a tentative hypothesis based on the existence of several L-arginine permeases with different characteristics. Two permeases would translocate arginine from outside to inside the cell. One anabolic permease would deliver the amino acid to the sites of protein synthesis, and a catabolic permease (when ammonium nitrate is absent) would deliver L-arginine to the site where arginase is located. A third permease, induced by arginine, would facilitate the communication of the two pools of L-arginine when ammonium nitrate is present. This hypothesis can be validated by a search for a mutant that only in the presence of ammonium nitrate utilizes exogenous arginine for catabolism.

By this token the preferential catabolism of exogenous over endogenous arginine, which occurs in the absence of ammonium nitrate, would be the result of the free operation of the catabolic permease of L-arginine and of the inactivity of the internal permease. The ability of *INO* to catabolize only endogenous arginine would be explained by the absence of the anabolic permease and by the inactivity of the catabolic permease for L-arginine: The only permease operating under these conditions would be the one which allows communication of the two pools. The different labeling of the arginine pools in *INO* and in *arg-8* in the absence of ammonium nitrate can be the result of a more or less complete segregation of the two arginine pools in the former mutant.

The segregation of biosynthetic from catabolic arginine in some way implies a segregation of the metabolites involved in these two pathways. Davis (14) has presented formal evidence that carbamyl phosphate, used for citrulline synthesis, segregates from carbamyl phosphate used for pyrimidine synthesis.

Recently, Mora and Davis performed some experiments to find out in a direct way if catabolic ornithine (ornithine produced by arginase) is carbamylated by ornithine transcarbamylase in *N. crassa*. In this work, three genetically different strains were used: 1) strain *arg-12* lacking ornithine transcarbamylase, and which is completely dependent on exogenous arginine to grow, 2) strain *arg-5* blocked in one of the steps of ornithine synthesis, and 3) normal, prototrophic strain 74A capable of endogenous arginine synthesis from the components of the minimal medium; this strain allowed us to estimate the extent of endogenous arginine synthesis in the presence of an excess of arginine in the medium. The analysis is based on the extent to which

the ^{14}C -guanido L-arginine provided in the medium is reduced in its specific radioactivity by the time it is incorporated into protein in these different strains. The reduction would come about by endogenous synthesis of ornithine or by the re-entry of catabolic ornithine via the ornithine transcarbamylase reaction, a reaction which would yield unlabeled citrulline and, ultimately, unlabeled arginine. The results presented in table 1 show that the specific radioactivity of the arginine found in proteins in strains 74A and *arg-5* is almost the same as that found in *arg-12*, indicating that, although the arginine is being catabolized, the ornithine liberated is not carbamylated at any great extent, and, consequently, that, at least under conditions of arginine excess, the ornithine cycle does not operate in *Neurospora*. This could be due to a feedback control of the production of ornithine and/or carbamyl phosphate, since the arginine added stops almost completely the synthesis of endogenous arginine in the strain 74A (table 1). Similar experiments previously reported (15) for the tryptophan cycle have shown that the dilution of this amino acid by the cycle amounts to 75%.

TABLE 1.—Growth of *Neurospora crassa* in the presence of ^{14}C -guanido L-arginine*

Strain	Sample	Dry weight ($\mu\text{g/ml}$)	Arginine in medium ($\mu\text{mole/ml}$)	Arginine in protein (specific radioactivity)
<i>Arg-5</i>	1	0.73	0.39	12,700
	4	1.80	0.02	13,800
<i>Arg-12</i>	1	0.73	0.37	13,700
	4	1.65	0.01	15,500
74A.....	1	0.4	0.62	12,350
	4	1.21	0.14	13,000

*Two hundred ml Vogel's medium in a 250 ml Florence flask containing 1 $\mu\text{mole/ml}$ of arginine with a specific radioactivity of 14,200 cpm/ μmole was inoculated to approximately 10^7 conidia per ml; vigorous aeration and mixing of the cultures were accomplished with a hydrated air line. The temperature was maintained at 25 C in a water bath. An aliquot was taken from the cultures and poured over a Millipore filter. The mold was scraped from the filter and put into cold 5% PCA. The residue was washed with cold PCA, resuspended in 3 N HCl and autoclaved at a pressure of 17 pounds. Arginine was purified from this hydrolysate by trapping it on a column of Dowex 50W. The arginine was eluted from the column and the specific radioactivity determined.

RESUMEN

Sa ha estudiado en el hongo *Neurospora crassa* el catabolismo de arginina biosintetizada endógenamente y agregada al medio de cultivo. Se ha encontrado que en este hongo la poza basal de arginina endógena no es asequible para catabolismo. La arginina endógena puede ser catabolizada solo cuando se aumenta la poza de este amino ácido; esto se logra administrando L-citrulina al medio de cultivo. Una poza elevada de arginina endógena y una arginasa, ornitino transaminasa y ureasa inducidas al máximo, no son condiciones suficientes que aseguren el catabolismo eficiente de este amino ácido; se requiere que el nitrato de amino este también presente en el medio de cultivo. Se ha encontrado una mutante de *N. crassa* que no

cataboliza arginina exógena cuando el nitrato de amonio está presente en el medio de cultivo. Experimentos realizados con guanido ^{14}C L-arginina, indican que en *N. crassa*, crecida en exceso de arginina, la ornitina producida por la acción hidrolítica de la arginasa no es un precursor de la arginina utilizada en la síntesis de proteína, por lo que se concluye que el ciclo de la ornitina es inoperante en este microorganismo.

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Characteristics of the Ureotelic Arginase and Its Role in the Advent of Ureotelism During the Metamorphosis of the Mexican Axolotl^{1,2,3}

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SUMMARY

It was found that the arginase in the liver of ureotelic animals differs from that in the liver of uricotelic animals and in *Neurospora crassa*. These two enzymes have similar properties. Induction of metamorphosis of the Mexican axolotl by administration of triiodotironine causes a shift from ammonotelism to ureotelism. This is accompanied by an increased capacity of the arginase activity already present to hydrolyze the arginase being produced from aspartic acid and citrulline; the addition of purified rat liver

arginase or purified axolotl liver arginase to a liver homogenate of an unmetamorphosed axolotl provokes the conversion of the arginine formed from citrulline and aspartic acid to ornithine and urea. Without these additions, arginine accumulates despite the fact that the arginase activity already present is able to hydrolyze arginine added to the incubation system. The implications of these findings relevant to the integration of a metabolic cycle are discussed.—Nat Cancer Inst Monogr 27: 283–295, 1967.

UREOTELISM has been established in higher organisms as a device for reducing the concentration of ammonia in cells and biological fluids to very low levels (1). The toxicity of this metabolite is such that a very efficient mechanism is needed to cope with the amount of ammonia that originates from the catabolism of nitrogenous compounds.

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² See Discussion of this paper conducted by Dr. R. L. Metzenberg, p. 000.

³ This investigation was supported by a grant (RF62005) from the Rockefeller Foundation.

⁴ We are indebted to Dr. Jaime Mora for his valuable tutoring of Miss Ortiz-Pineda through a major part of the work and to Professor Philip P. Cohen and Dr. Jaime Martuscelli for their encouragement and criticism.

Five enzymes that constitute a metabolic cycle participate in the formation of urea. Several of them are found in lower organisms in the biosynthetic path that leads to the production of arginine. However, in ureotelic animals the synthesis of carbamyl phosphate, a precursor of arginine and subsequently of urea, is carried out by an enzyme different from the one that intervenes in lower organisms in the synthesis of carbamyl phosphate, a precursor of arginine for protein synthesis. The former enzyme has a lower K_m for ammonia and requires 2 moles of ATP and *N*-acetyl glutamate (2). The fungal and bacterial carbamyl phosphate synthetases generally appear not to require *N*-acetyl glutamate, but they will utilize the γ -amide nitrogen of glutamine as a nitrogen source for the carbamyl group, and will alternatively use ammonium ions at a high concentration. A reaction which can form carbamyl phosphate from a single equivalent of ATP (the "carbamate kinase" reaction) is probably purely catabolic *in vivo*.

The metabolic cycle is integrated by the addition of arginase, which hydrolyzes arginine to give urea and ornithine. The ornithine is reutilized for the synthesis of citrulline by coupling with the carbamyl moiety of carbamyl phosphate (2).

It was assumed that an investigation of the physicochemical characteristics of arginase and of its possible role in intermediary metabolism in species varying in their nitrogen excretory pattern would throw light on how a metabolic cycle starts its operation for the performance of a new function. Because of reasons given below, the transition from ammonotelism to ureotelism in the metamorphosing Mexican axolotl adds a new dimension to this particular problem. This biological model also provides a unique opportunity to observe the physical and functional integration of a metabolic cycle.

TYPES OF ARGINASES AND THEIR PROPERTIES

Arginase is present in very primitive biological forms. It has been described in *Bacillus licheniformis* (3, 4) and in *Neurospora crassa* (5). It also exists in several invertebrates, and in some of these it has been implicated in the biosynthesis of urea (6-11). It is present in the liver of ureotelic vertebrates together with the other enzymes that participate in the ornithine cycle, and its presence has been traced back to primitive fishes (12).

For a time it was believed that arginase was absent from the liver of uricotelic animals. This belief was commonly known as the rule of Clementi (13). However, although recent work has demonstrated a low arginase activity in chicken liver (14, 15) and in other uricotelic species (16), it has been proven that this enzyme is a different protein

from that occurring in the liver of ureotelic animals (17). They differ in several respects: (a) K_m , (b) inhibition by an excess of substrate, (c) antigenicity, (d) molecular weight, (e) stability during dialysis, and (f) inhibition by *p*-chloromercuribenzoate. In addition, they behave differently when submitted to electrophoresis in acrylamide gel, and they are purified by a different procedure (unpublished results). Table 1, which illustrates these differences, also includes the characteristics of the arginase of *Neurospora crassa* (18). It can readily be seen that the *Neurospora* enzyme resembles very closely the "uricotelic" arginase.

With respect to the arginase found in *N. crassa*, it is opportune to mention that the existence of the Krebs-Henseleit cycle in fungi was postulated because of the presence of arginine biosynthetic enzymes and arginase (5). However, this thesis was challenged on the grounds that urease activity was also present, that a high ammonia pool existed in the microorganisms, that arginine is not an indispensable amino acid for *N. crassa*, and because of the existence of a mutant, arg-8, which is an auxotroph for proline and grows when arginine or citrulline is present in the medium (19, 20). It was demonstrated later that the arginase in *N. crassa* can hydrolyze only exogenous arginine and not endogenous arginine, *i.e.*, that produced from citrulline and aspartic acid (19, 20). This fact might be relevant to the metabolic role of the "uricotelic" arginase.

Cabello has reported two molecular forms of arginase in human liver and erythrocytes (21), but it is not clear whether they bear any relation to the other arginases studied in our laboratory.

Of great interest is the finding of a high arginase activity of the "ureotelic" type in the liver of the uricotelic terrestrial turtle (*Gopherus berlandieri*). The presence of this enzyme was interpreted as a vestige from the turtle's ureotelic past. Recently, Brown reported that the activity of the arginase in the liver of the seagull is unusually high as compared with the levels commonly observed in uricotelic species, and interestingly enough, it is clearly inhibited by substrate (22). If this enzyme also proves to be "ureotelic," its existence might be explained as in the case of the uricotelic turtle.

All these findings allow us to revise Clementi's rule as follows: There is no "ureotelic" arginase in the liver of uricotelic animals. The exception to the rule is the enzyme that exists as a remnant from a previous (in evolutionary terms) ureotelic habit. It has also been postulated that a further mutation in those uricotelic species having "ureotelic" arginase leading to the deletion of the latter enzyme would not handicap them for survival (16).

Conversely, to function in the biosynthesis of urea, arginase should be of the "ureotelic" type.

TABLE 1.—Properties of arginases from different sources

Source of arginase	Activity	K_M	Inhibition by substrate	Precipitation with antibody against purified rat liver arginase*	Molecular weight	Stability during dialysis	Inhibition by 5×10^{-3} M <i>p</i> -CMB	Specificity†	Ratio of hydrolysis L-arginine/L-canavanine
Rat liver.....	High	10^{-2} M	+	+	138,000	Stable	0	Only L-arginine and L-canavanine are substrates	1.5
Chicken liver.....	Low	10^{-1} M	None	—	276,000	Unstable	100%	Only L-arginine and L-canavanine are substrates	7.2
<i>Neurospora crassa</i>	Low (inducible)	10^{-1} M	None	—	270,000	Unstable	100%	Only L-arginine and L-canavanine are substrates	6.5

*Precipitation was with rabbit antibody to purified rat liver arginase. This antibody was kindly supplied by Dr. Robert T. Schimke, National Institutes of Health, Bethesda, Md.
†D-Arginine, guanido acetic, guanido propionic, guanido butyric, and methyl guanidine were also tested.

CAPACITY OF THE UREOTELIC ARGINASE TO HYDROLYZE ENDOGENOUS ARGININE

The presence of "ureotelic" arginase, in addition to carbamyl phosphate synthetase, ornithine transcarbamylase, arginino succinase, and arginino succinase, is not enough to integrate the Krebs-Henseleit cycle. This fact became apparent while the development of the urea biosynthesis enzymes was being studied during the metamorphosis of the neotenic amphibia, the Mexican axolotl (*Ambystoma mexicanum*). This animal reproduces in the larval stage, which is aquatic, and excretes most of the nitrogen from its catabolism as NH_3 (60–80%), the remainder being accounted for mainly as urea (20–40%) (23). The axolotl can be induced to metamorphose by the administration of thyroid hormones, and when this happens it becomes ureotelic (23).

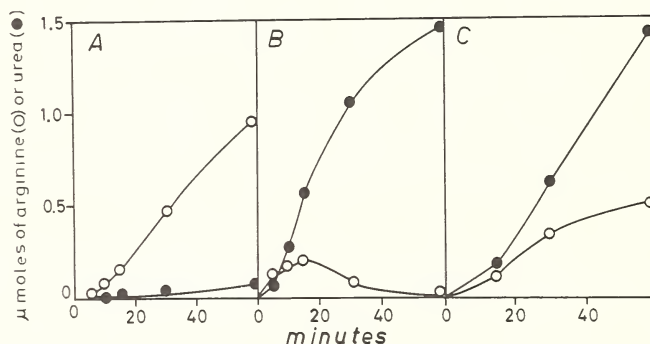
It has been suggested that the advent of ureotelism during the metamorphosis of the tadpole can be explained by a great increase (15-fold–30-fold) in the activity of all urea cycle enzymes (24), and it has been proved in an elegant manner that the augmentation in carbamyl phosphate synthetase corresponds to synthesis *de novo* (25). However, metamorphosis in the Mexican axolotl is accompanied by only a slight increase in carbamyl phosphate synthetase and ornithine transcarbamylase and by none whatsoever in arginine synthetase and arginase (16).

When the arginine synthetase activity (arginine succinate synthetase plus arginino succinase) was originally measured in the liver of the unmetamorphosed Mexican axolotl, none was detected (26). But when this experiment was repeated, it was realized that arginine synthetase was indeed present and that it was not observed before because of the nature of the assay system. It has been assumed that the high arginase activity already demonstrated to be present would readily hydrolyze whatever arginine was produced from citrulline, aspartic acid, and ATP, allowing the formation of urea as a function of time to serve as the indication of the rate of arginine synthesis. It was found that, as the reaction proceeded, a compound accumulated in the incubation system which produced the characteristic color given by arginine in the Sakaguchi method. If the reaction was stopped with trichloroacetic acid, and the mixture extracted with ether and then neutralized, the addition of purified arginase caused the compound to disappear. The compound had the same R_f as arginine when chromatographed in two different solvent systems.

The same compound accumulated when rat liver homogenate was incubated under identical conditions but with isoleucine added to inhibit arginase. It was concluded that the compound was indeed arginine, and that it accumulated because somehow the arginase al-

ready present in the liver of the Mexican axolotl was not able to hydrolyze it, even though it could split exogenous arginine (added to the incubation system) to urea and ornithine (16).

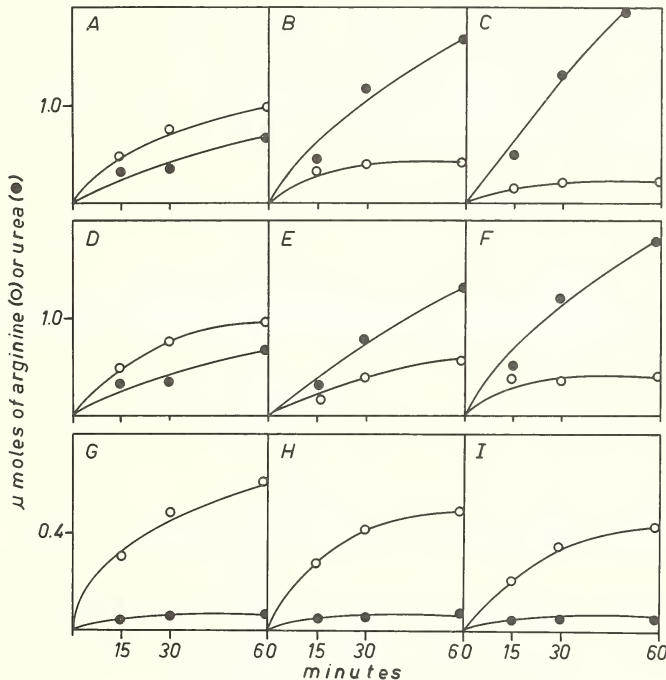
Text-figure 1 shows that an axolotl in which metamorphosis had been induced by intraperitoneal injection of triiodothyronine had an enhanced capacity to hydrolyze endogenous arginine. Even though the metamorphosed axolotl's efficiency to conversion of arginine to urea is not as high as that seen in the rat, there is a striking difference between its situation and that prevailing in the unmetamorphosed axolotl, where arginine accumulates and very little urea is formed. In fact, the capacity of the metamorphosed axolotl resembles that observed in the frog, which is also ureotelic (16). The participation of an inhibitor was ruled out by appropriate mixing of axolotl liver homogenates and by addition of a heated homogenate from axolotl liver to a fresh rat liver preparation.



TEXT-FIGURE 1.—Conversion of endogenous arginine into urea in the livers of the unmetamorphosed (A) and metamorphosed (C) Mexican axolotl as compared with that in the liver of the rat (B). The incubation system contained 40 mg wet weight of liver tissue, 5 μ moles each of L-citrulline, aspartic acid, ATP and MgSO_4 , and 50 μ moles of potassium phosphate buffer, pH 7.0, in a final volume of 1 ml. Taken from Mora *et al.* (16).

It was then assumed that the arginase in the liver of the unmetamorphosed Mexican axolotl is not capable of splitting endogenously produced arginine for one of the following reasons: (a) either the endogenous arginine pool is separated from arginase by a physical barrier, or (b) arginase is located in a compartment that does not allow integration of its function with those functions of other enzymes of the urea cycle. The latter reason can be due to conformational changes of the protein molecule or to its binding to a given component that hampers its association with the corresponding protein aggregates (*see* section on Physical Integration of a Metabolic Cycle). What-

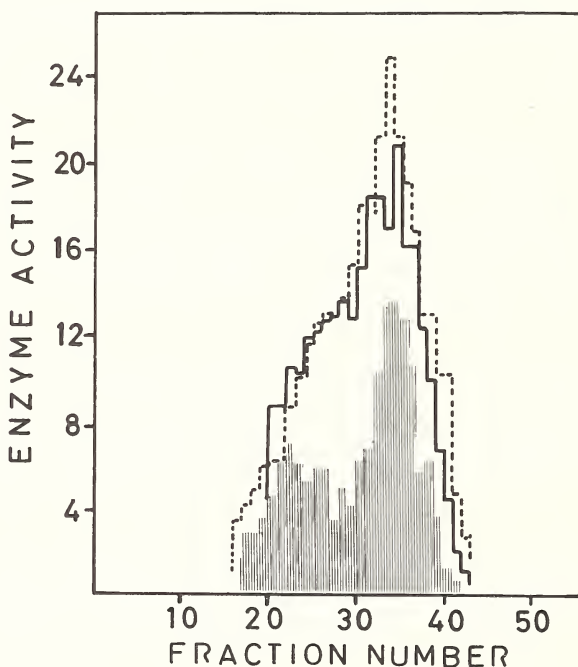
ever the case, it is clear that the catalytic site of the enzyme is not altered, since it is capable of splitting exogenous arginine. Although this problem is not yet solved, the experiments illustrated in text-figure 2 give support to possibility (b). It can be seen that the addition of purified rat or axolotl liver arginases to liver homogenates from an unmetamorphosed axolotl (therefore originally unable to hydrolyze endogenous arginine) renders this system capable of urea formation from citrulline and aspartic acid. Interestingly, the hydrolysis of the endogenous arginine cannot be accounted for by the activity contributed by the purified arginases, which with purified



TEXT-FIGURE 2.—Conversion of endogenous arginine into urea by liver homogenates of unmetamorphosed axolotl to which was added purified “ureotelic” arginase from rat and unmetamorphosed axolotl or purified “uricotelic” arginase from *N. crassa*. The systems were set as follows: A, D, and G contain liver homogenates from unmetamorphosed Mexican axolotl as the only source of enzyme; B and C are the same as A plus 10 and 20 units of purified rat liver arginase, respectively; E and F are the same as D plus 0.6 and 1.2 units of purified unmetamorphosed axolotl arginase, respectively; H and I are the same as G plus 70 and 105 units of purified *N. crassa* arginase, respectively. A and D are duplicates of the same homogenate. The “ureotelic” arginases were purified following the instructions given by Schimke *et al.* (27), and the “uricotelic” arginase as described by Mora *et al.* (18). The experimental conditions were as indicated in text-figure 1.

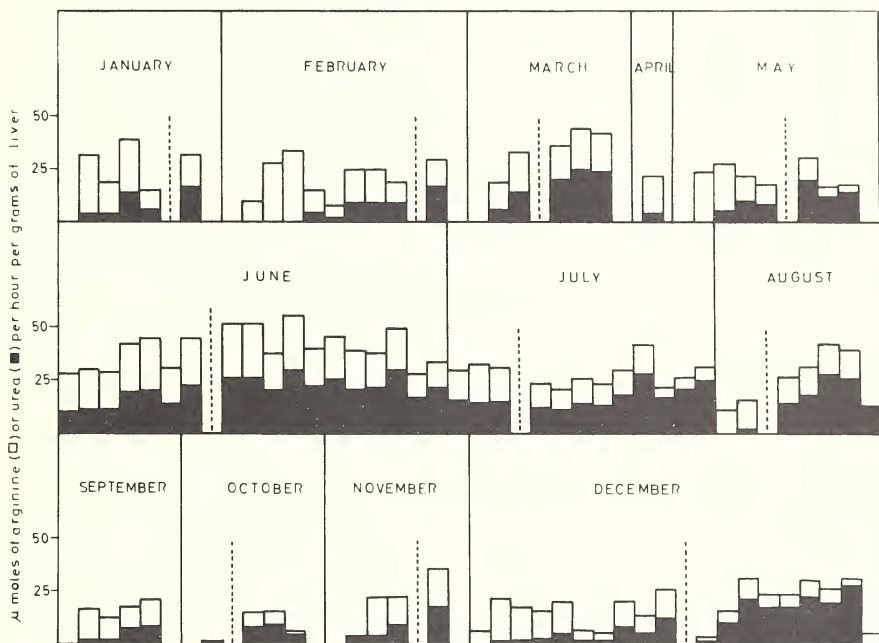
axolotl arginase is negligible under the suboptimal conditions of the incubation system (pH 7.0, no Mn^{++} added). The addition of the purified arginase from *N. crassa* did not have the same effect, even though much more activity was included.

It was observed that the arginase activity in the liver of the unmetamorphosed axolotl can be separated into two components by centrifugation in a density sucrose gradient, but the activity in the liver of the metamorphosed animal shows only one component and exactly the same pattern as that given by the rat liver arginase (text-fig. 3). A concentration of 5×10^{-3} M of *p*-chloromercuribenzoate, which suppresses completely the activity of the "uricotelic" arginases but does not affect the "ureotelic" arginase, inhibits 30–40% the arginase activity of a crude homogenate from unmetamorphosed Mexican axolotl. This observation might have some relation to the presence of the arginase peaks already mentioned.



TEXT-FIGURE 3.—Sucrose gradient analysis (carried out by Miss Cristina González-Navarro) of ureotelic arginase from rat liver (*broken line*), metamorphosed (*continuous line*), and unmetamorphosed (*shadowed area*) axolotl liver. Conditions of the assay: The preparations were centrifuged at $100,000 \times g$ for 16 hours. Enzyme activity is expressed as μ moles of substrate converted into product/hour/0.094 ml of effluent.

Apparently, there is a seasonal variation in the efficiency of the axolotl arginase for hydrolysis of endogenous arginine. For example, a greater capacity in the summer months is noticeable (text-fig. 4). Also, from September to December, the activity of the arginine synthetase system is lowered as judged by the total amount of arginine plus urea. There is not a clear correlation between the efficiency of arginase for hydrolysis of endogenous arginine and urea excretion.



TEXT-FIGURE 4.—Efficiency of the conversion of endogenous arginine into urea by the liver homogenates of unmetamorphosed Mexican axolotl during various months of the year. Each vertical bar represents a different animal; to the right of the broken line are included those cases that produce more urea than arginine. The experimental conditions were as indicated in text-figure 1.

PHYSICAL INTEGRATION OF A METABOLIC CYCLE

It can be predicted that some sort of physical connection should be established between enzymes functioning as a multienzyme system in a given metabolic path. This type of organization is necessary for the channeling of the metabolites handled by the metabolic path. There are several well-documented cases that illustrate what may prove to be the general case. The enzymes described by Lynen (28), which participate in the biosynthesis of long-chain fatty acids, are linked one

to the other to form a group that behaves as a single entity. The protein aggregate has been crystallized and has recently been observed by electron microscopy (29). The same has been achieved with the pyruvate dehydrogenase complex and its component lipoyl reductase-transacetylase (30) and with the lipoyl reductase-transsuccinylase aggregate from the α -ketoglutarate dehydrogenase complex (31). Two different proteins constitute the tryptophan synthetase system; each has a different catalytic activity, and the summation of them converts indole-glycerol phosphate and L-serine to L-tryptophan and glyceraldehyde-3-phosphate (32). Wagner *et al.* have demonstrated that, for the synthesis of isoleucine and valine, not only is the presence of the intervening enzymes necessary, but also their physical integration (33).

Therefore, it would be reasonable to predict that the urea cycle enzymes should also be linked into a functional unit. However, as previously reported (15) this postulate cannot be proved by differential centrifugation (table 2), and the data collected pose a difficult problem.

TABLE 2.—Differential centrifugation of liver homogenates from rat and nonmetamorphosed Mexican axolotl*

Fraction	Carbamyl phosphate synthetase		Ornithine transcarbamylase		Arginine synthetase system		Arginase	
	Rat	Axolotl	Rat	Axolotl	Rat	Axolotl	Rat	Axolotl
Nuclei.....	34	9	41	37	0	0	40	3
Mitochondria.....	66	44	53	32	0	0	19	1
Microsomes.....	0	0	2	0	0	0	37	3
Supernatant.....	0	47	4	31	100	100	4	93

*Numbers express the percentage of the total activity present in each fraction.

Indeed, carbamyl phosphate synthetase and ornithine transcarbamylase are located mainly in mitochondria, whereas the arginine synthetase system resides in the supernatant and arginase is distributed among nuclei, mitochondria, and microsomes. This would mean that the citrulline synthesized in mitochondria by the first two enzymes has to get to the proper place in the soluble fraction to be converted to arginine, which in turn has to find its way to the endoplasmic reticulum and/or other particles to come in contact with arginase. Following the hydrolysis of arginine, urea must be excreted and ornithine must again be transported to mitochondria. It is difficult to believe that this situation exists inside the cell, and the very low concentrations of arginine and ornithine in the liver cell (34) do not support this type of reasoning.

The explanation might be that either the representation derived from the differential centrifugation data does not correspond to the location that really exists in the intact cell or that the enzymes are located as they were found, but some mechanism of integration among them escapes our present understanding. The first situation might be produced by the leakage of the enzymes from their original aggregate, because the protein interaction is disrupted prior to or during differential centrifugation. Following this, the proteins might become adsorbed to particles with which they would not be associated *in vivo*.

In relation to the findings recorded in table 2 for the unmetamorphosed Mexican axolotl, it is of great interest that, in addition to the first two enzymes of the cycle being less strongly associated with particles than in the rat, arginase clearly remains soluble and is not significantly attached to any particle. This observation seems to contribute further evidence to the theory that the arginase molecule of the unmetamorphosed axolotl is not associated with the proper structure. Studies on the differential centrifugation of liver homogenates from the metamorphosed animal and on the distribution of the purified enzyme, when added to crude homogenates from rat and axolotl livers, are in progress.

FUNCTIONAL INTEGRATION OF METABOLIC CYCLE

If the integration of carbamyl phosphate synthetase and the ureotelic arginase with the other enzymes of biosynthesis of arginine results in the establishment of a functional unit for the formation of urea (the Krebs-Henseleit cycle), then arginine may become partly or completely unavailable for protein biosynthesis. Accordingly, it is not fortuitous that arginine is an indispensable amino acid for the rat, a fully ureotelic animal (35). Even in the case of the human, where arginine was originally classified as nonessential, the amino acid is apparently necessary for optimal growth (36).

The Mexican axolotl should provide a suitable system for looking into the availability of the amino acid for protein synthesis as an animal progressively shifts toward the formation of urea. The possibility of inducing a controlled metamorphosis by the use of thyroid hormones adds another controllable parameter to the system.

RESUMEN

La arginasa presente en el hígado de los animales ureotélicos es una proteína distinta de aquella existente en el hígado de los animales uricotélicos y en *Neurospora crassa*. Estas dos últimas enzimas tienen propiedades similares. La inducción

de la metamorfosis en el ajolote Mexicano por triodo-tironina se acompaña de un aumento en la capacidad de la arginasa hepática en hidrolizar la arginina que se produce a partir de citrulina y de ácido aspártico. Si se añade arginasa purificada de hígado de rata o arginasa purificada de hígado de ajolote a un homogeneizado de hígado de ajolote metamorfoseado, la arginasa formada de citrulina y ácido aspártico se convierte en ornitina y urea. La arginasa presente con anterioridad en el homogeneizado no efectúa esta conversión a pesar de que es capaz de hidrolizar la arginina añadida al sistema de incubación se discute la importancia de estos resultados sobre la integración física y funcional de un ciclo metabólico.

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DISCUSSION¹

Discussor, DR. R. L. METZENBERG, Department of Physiological Chemistry, The University of Wisconsin, Madison, Wisconsin

DR. PARDEE was asked whether his model of sulfate transport required that a phosphate from ATP hydrolysis be rejected each time a sulfate ion was transported into the cell. He responded that this was implied by the model, but that it was easy to construct models in which all hydrolytic products are retained by the cell. He further suggested a possible analogy between the sulfate carrier protein and the product of the R2 gene for control of alkaline phosphatase in *E. coli*, which could conceivably be a phosphate carrier protein.² In answer to another question, he noted that three different cistrons seem to be involved in sulfate transport, and that the total picture may be quite complicated.

Dr. Cabello, in commenting on the paper presented by Drs. Soberón, Ortíz-Pineda, and Tarrab, noted that an arginine auxotroph of *Neurospora crassa*, growing on limiting arginine as a sole nitrogen source, exhibited a sudden increase in arginase upon entering the exponential growth phase. Presumably this has the effect of liberating urea, and, via urease, ammonia nitrogen for the synthesis of other amino acids. This should be contrasted with the "ureotelic" arginases that are primarily involved in the detoxification of ammonia, rather than its production. Dr. Cabello also presented findings showing that arginase which is modified by sodium dodecyl sulfate exhibits a sigmoidal kinetic curve as a function of arginine concentration, rather than the usual rectangular hyperbola. He suggested that natural analogues of dodecyl sulfate, perhaps salts of fatty acids, may modify the properties of arginase *in vivo*, and that such conversions might be the basis of the changes observed in the axolotl by Soberón and his colleagues.

¹ Of articles by Arthur B. Pardee; José L. Reissig, Abel S. Issaly, and Inda M. de Issaly; L. Cañedo, J. Martuscelli, and J. Mora; Guillermo Soberón, Juana Ortíz-Pineda, and Rebeca Tarrab.

² Garen, A., and Otsuji, N.: *J Molec Biol* 8: 841, 1964.

Because of the complexity of the subject matter of compartmentalization and channeling of metabolites, it was very difficult to deal with these problems in front of a large audience; many people approached Drs. Reissig and Mora with private questions after the talks. It is unfortunate that the ensuing discussions are not available for this record.

THURSDAY MORNING

Chairman: **Jesús Kumate**

Protein Turnover and the Regulation of Enzyme Levels in Rat Liver^{1,2}

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SUMMARY

As opposed to logarithmically growing bacteria, there is an extensive, continual synthesis and degradation (turnover) of total protein, as well as specific enzymes, in animal tissues. An observed enzyme level, then, is the summation of both processes of synthesis and degradation, each of which can be altered independently by changes in physiological and nutritional state, or by the administration of hormones or other compounds. In the case of tryptophan pyrrolase, glucocorticoid administration increases the enzyme level as a result of an increased rate of enzyme synthesis, whereas the substrate-induced accumulation of enzyme can be attributed to a cessation of enzyme degradation, *i.e.*, enzyme stabilization, in the presence of continued enzyme synthesis. There is a marked heterogeneity of turnover rates of different enzymes in rat liver, with half-lives

that vary from several hours to 4–5 days. The levels of enzymes with rapid turnover rates will be more responsive (change in the fold level of enzyme) to altered rates of synthesis or degradation, than those with a slow turnover. Thus cortisone administration increases the rate of synthesis of tryptophan pyrrolase, glutamate-alanine transaminase, and arginase to approximately the same extent. However, the time courses of increase are markedly different because of different turnover rates of these enzymes. The mechanism(s) and regulation of events involved in enzyme degradation are not well understood at present. Agents or drugs which inhibit energy metabolism and protein synthesis also inhibit protein degradation. Thus the process of protein breakdown is complex and is altered by multiple factors. —*Nat Cancer Inst Monogr* 27: 301–314, 1967.

THIS PAPER briefly describes some studies from the author's laboratory on the interplay of both enzyme synthesis and degradation in the control of enzyme levels in animal tissues, most specifically in rat liver.

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

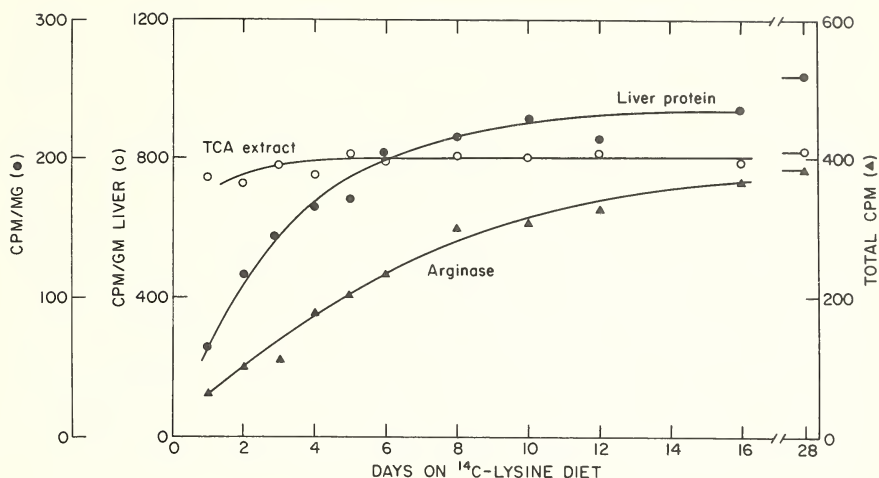
² See Discussion of this paper conducted by Dr. G. D. Novelli, p. 325.

Recent knowledge of cellular regulatory mechanisms (1, 2) has been obtained largely from studies with microorganisms, most commonly *Escherichia coli*. Such a unicellular organism, selected for rapid growth under a variety of nutritional conditions, is vastly different from the individual cell in a multicellular organism. This latter cell is often not growing, may carry out a highly specialized function, and is associated with similar and dissimilar cells in tissues and organs. In such a different "biosphere," new regulatory problems have arisen, and new solutions have been found or superimposed on basic regulatory mechanisms common to all organisms. One such "solution" has been the elaboration of a variety of hormones necessary for the integrated functioning and development of various tissues and organs. Another solution can involve the continual synthesis and degradation of intracellular protein. The problem in this case would be how to effect changes in metabolic machinery in response to environmental and nutritional changes, a process that would include removal of unneeded enzymes as well as the synthesis of those newly required. In bacteria the removal process can involve dilution during phases of rapid growth. Only under nongrowing conditions does demonstrable protein turnover occur in bacteria (3). In animal tissues, on the other hand, where no growth and little cellular division take place, the process of protein degradation becomes increasingly more significant as a means of removing unneeded metabolic machinery. It is of interest that the rate of protein turnover is greatest in liver, the organ whose enzyme profile undergoes many changes under various physiological and nutritional conditions.

TURNOVER OF TOTAL LIVER PROTEIN AND ARGINASE

To understand some of the ways in which protein turnover is significant in the regulation of enzyme levels, certain information about the general process of protein turnover in liver would be helpful. Text-figure 1 shows an experiment designed to answer questions about the rate and extent of turnover of total liver protein and one specific protein, arginase (4).

In this experiment rats were fed an amino acid diet containing lysine of a known, constant specific activity for up to 28 days. The rate and extent of protein turnover were estimated from how rapidly and to what extent the cellular protein and arginase were replaced from the dietary source. The incorporation of lysine into total liver protein was initially rapid, and then slowed markedly after 5-6 days. The incorporation of lysine into arginase was slower. This demonstrates the first important property of protein turnover in liver. There is not one rate of turnover, but different rates for each protein. Similar



TEXT-FIGURE 1.—Incorporation of continuously administered ^{14}C -L-lysine into total protein, arginase, and trichloroacetic acid (TCA) soluble extracts of rat liver. Osborne-Mendel rats maintained for 7 days on a diet consisting of 25% complete amino acid mixture were then placed on a similar diet containing ^{14}C -L-lysine. At intervals one rat was killed; the liver was removed and divided into two portions. One sample was made into acetone powder, from which arginase was isolated by immunological techniques; the other was treated with 10% trichloroacetic acid and divided into protein and supernatant fractions. Radioactivity of the trichloroacetic acid soluble counts is expressed as counts per minute per extract from 1 g of liver, wet weight (○—○). Counts in total liver protein: counts per minute per mg of protein (●—●). Counts in the arginase: total counts precipitated (▲—▲) (4).

findings have been made by Swick (5) and Buchanan (6). One group of proteins has a mean half-life of about 2–3 days, whereas others have half-lives in the range of 20–60 days. As will be discussed later, some enzymes have half-lives of the order of several hours.

This experiment also allows for a measure of the extent to which cellular proteins are replaced. Thus, the ^{14}C -lysine incorporation may represent anywhere from 1–100% replacement of the lysine residues of the protein. An answer to this question can be obtained by comparing the specific activity of the lysine of the protein with that of the dietary source. Accordingly, samples of total liver protein, as well as arginase purified by standard purification procedures, were obtained after 20 days of continuous dietary labeling. Protein samples were hydrolyzed, and the lysine was isolated by column chromatography. After 20 days of labeling approximately 75% of the lysine residues of total liver protein, and virtually all of the lysine of arginase had been replaced (4).

On the basis of these studies, certain properties of protein turnover can be generalized: 1) The replacement of protein in rat liver is exten-

sive and rapid. Fifty percent is replaced every 4–5 hours. The turnover does not involve only the proteins synthesized and secreted by the liver. A major protein secreted by the liver is albumin. The steady-state amount of albumin in liver has been estimated to be 1–2% (7). Thus, if ^{14}C -lysine had been incorporated only into albumin, the final specific activity of ^{14}C -lysine would have been only 1–2% of that of the dietary protein, whereas after 20 days it was in fact 76%. 2) The majority of the protein turnover is intracellular, rather than involving replacement of cells. This statement is based on investigations which show that the lifespan of cells in liver is from 160–400 days (6, 8, 9). It follows that since the majority of liver protein is replaced within 20 days, the turnover that occurs must be largely intracellular. 3) There is a marked heterogeneity of turnover rates for different proteins; the half-lives vary from several hours to at least 60 days.

TURNOVER RATES OF SPECIFIC LIVER ENZYMES

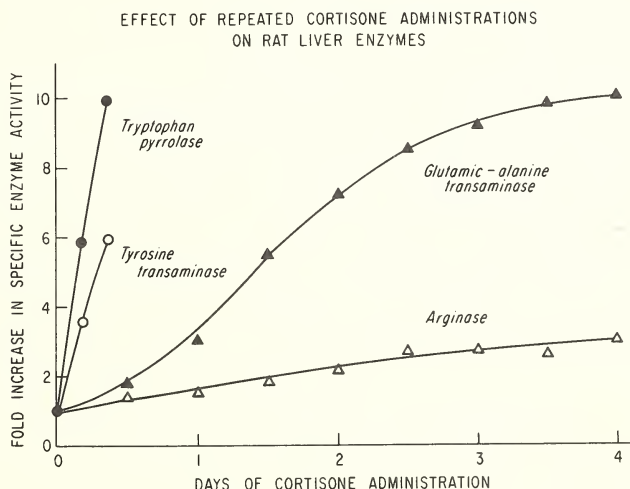
Information on turnover rates of specific enzymes is extremely limited, a situation due in part to problems involved in obtaining valid measurements. A variety of methods have been employed, each with certain assumptions and limitations. For instance, the decay of enzyme activity after an initial increase to high levels by administration of a glucocorticoid has been used to obtain half-lives of 2–3 days for glutamic-alanine transaminase (10) and 2–3 hours for tryptophan pyrrolase (11) and tyrosine transaminase (12). The decay of enzyme activity after prevention of further protein synthesis by puromycin administration has given half-lives of 2–4 hours for tryptophan pyrrolase (13) and 75 minutes for δ -aminolevulinic acid synthetase (14). By use of an irreversible inhibitor of catalase, a half-life of 24 hours was obtained (15). The most desirable half-life, of course, is that obtained under basal conditions. Each of the above methods involves administration of agents that may themselves affect rates of degradation. They also involve the assumption that loss of enzyme activity under the conditions employed reflects rates of synthesis and degradation under basal, steady-state conditions.

Rates of turnover of arginase, tryptophan pyrrolase, and tyrosine transaminase under basal conditions have been determined by use of combined immunologic and isotopic techniques. Arginase has a half-life of 4–5 days (4). Values obtained for tyrosine transaminase (16) and tryptophan pyrrolase (17) of approximately 2–4 hours are similar to those obtained by less rigorous techniques.

EFFECTS OF TURNOVER RATES ON APPARENT RESPONSE OF ENZYMES TO CORTISONE

The heterogeneity of turnover rates of individual enzymes is of significance in considering the specificity of response to agents which increase rates of enzyme synthesis. Thus, there are a large number of enzymes in rat liver which are increased in quantity following corticosteroid administration, and in which an increase in rate of enzyme synthesis has been implicated (18). For instance, tryptophan pyrrolase and tyrosine transaminase respond with fourfold to eightfold increases 4 hours after a single administration of cortisone, whereas glutamic-alanine transaminase and arginase increase very little. Such results suggest that cortisone specifically stimulates the synthesis of tryptophan pyrrolase and tyrosine transaminase. However, this apparent specificity can be explained by the marked differences in rates of turnover of these enzymes, with cortisone stimulating the synthesis of each to approximately the same extent.

As shown in text-figure 2, when cortisone is administered repeatedly for 4 days, it does indeed increase the levels of arginase and glutamic-alanine transaminase, as well as tryptophan pyrrolase and tyrosine transaminase. From the data in this text-figure, from knowledge of the half-lives of these enzymes, and from the basal amount of enzyme, estimates of the relative effects of cortisone in increasing rates of synthesis of all four enzymes can be made as shown in table 1. In the



TEXT-FIGURE 2.—Time course of the increase in tryptophan pyrrolase, tyrosine-glutamic transaminase, glutamic-alanine transaminase, and arginase with cortisone administration. Animals received 10 mg of cortisone acetate intramuscularly every 8 hours. Each value is the mean of 3 animals (12).

first column are given the half-lives of the enzymes as obtained by a variety of previously discussed techniques. In the second column are the enzyme levels under basal conditions. From the known half-life and from the basal level of enzyme, the rate of synthesis can be calculated (third column). The rate of synthesis during cortisone administration is taken from the most nearly linear portion of the accumulation of enzyme activity shown in text-figure 2 (fourth column). In the last column are the ratios of rates of synthesis with cortisone to those under basal conditions for the four enzymes. These ratios are very similar, varying from 4-7. Thus, in spite of the marked differences in response to cortisone of the enzyme levels, the rates of synthesis of the four enzymes were increased to essentially the same extent.

TABLE 1.—Comparison of rates of enzyme synthesis under basal conditions and during cortisone treatment (12)

Enzyme	Half-life (hrs)*	Basal enzyme activity (units)†	Enzyme synthesized		Ratio cortisone: normal
			Basal (units/hr)	Cortisone (units/hr)	
Tryptophan pyrrolase.....	2.5	3.0	.84	3.4	4.0
Tyrosine-glutamic transaminase..	2.0	78	27	114	4.2
Glutamic-alanine transaminase..	84	252	2.0	14.4	7.2
Arginase.....	96	13,800	138	534	3.9

*The relationship between the half-life ($t_{1/2}$) and the value k used for these calculations is given by $t_{1/2} = \frac{\ln 2}{k}$.

(See footnote 4, p. 307, for method of calculation.)

† Enzyme unit/g wet weight.

The apparent specificity of response of tryptophan pyrrolase and tyrosine transaminase, then, can be considered to be primarily a reflection of the higher rate of turnover, rather than a specific stimulation of the rate of synthesis of these two enzymes. It is of interest that all four of these enzymes involved in gluconeogenesis are affected in the same manner and to the same extent. Perhaps the functional unit that controls the synthesis of a number of enzymes involved in gluconeogenesis is controlled by a single mechanism, and acts operationally as an "operon" in the classical bacterial sense (1).

SYNTHESIS AND DEGRADATION OF TRYPTOPHAN PYRROLASE³

It is apparent from the preceding section that when the rate of synthesis is changed the levels of enzymes with rapid rates of turnover

³ As used here, the two words "inactivation" and "degradation" cannot be distinguished operationally. Thus, it is impossible to detect a protein as a given enzyme once it has irreversibly lost enzyme activity or lost immunologic reactivity. It can only be inferred that the inactive (unrecognizable) product is eventually degraded.

can be changed more rapidly and dramatically than those with little turnover. In addition, the level of an enzyme with a rapid turnover rate can be regulated by the rate at which the enzyme is degraded or irreversibly inactivated.⁴ The following studies with tryptophan pyrrolase indicate how the processes of synthesis and degradation interact to determine the given enzyme level (17). This enzyme catalyzes the conversion of tryptophan to formylkynurenine and contains a readily dissociable prosthetic group, hematin. From the studies of Knox (20, 21) and Feigelson and Greengard (22, 23), it has been known that enzyme activity is increased by the administration of either hydrocortisone or tryptophan, but that the mechanisms for the two types of induction, both resulting in new enzyme synthesis (24), are different. Because previously discussed studies indicated a rapid turnover of the enzyme, we undertook a series of studies and found that the hormonal induction of the enzyme results from an increased rate of enzyme synthesis, whereas the substrate effect results in the maintenance of the enzyme in an active form not susceptible to the degradation process, *i.e.*, stabilization. It is this latter point that I wish to emphasize.

Critical evidence in support of the concept that tryptophan administered to an animal prevents breakdown of the active, immunologically reactive tryptophan pyrrolase is shown in text-figure 3 (17). Enzyme was prelabeled by administration of ¹⁴C-leucine 60 minutes before the time indicated as zero. Forty minutes after the administration of a single dose of leucine, no more incorporation of radioactivity occurs into total liver protein. Therefore, any protein synthesized after that time will be derived from unlabeled leucine. Although the amount of enzyme activity remained constant over a subsequent 9-hour period, the radioactivity present in prelabeled tryptophan pyrrolase isolated by immunologic techniques diminished progressively. This, then, is

⁴ The most simplified model for a change in tissue level of an enzyme involving both enzyme synthesis and degradation is:

$$dP/dt = S - kP \quad [1]$$

where P is the content of enzyme per unit weight, S the rate constant for synthesis, expressed as enzyme units/time, and k the first-order rate constant for enzyme degradation, expressed as time⁻¹. In animal tissues a change in total liver mass can generally be ignored. At any time that a steady state for an enzyme level exists, *i.e.*, $dP/dt = 0$, then

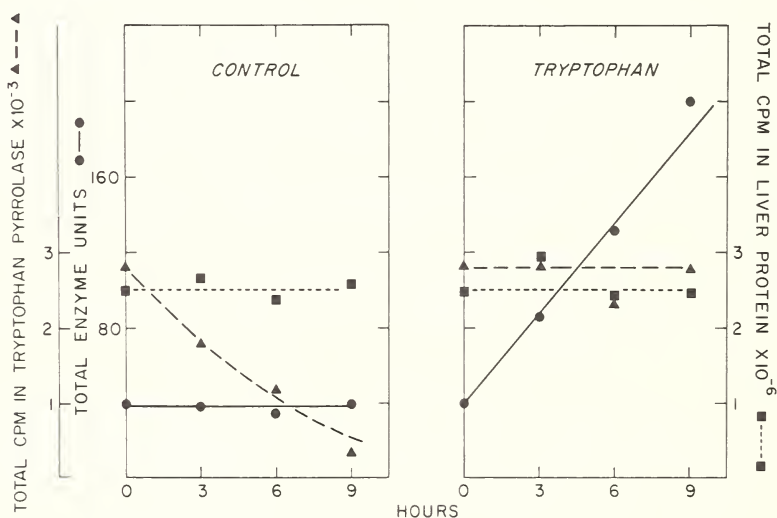
$$P = S/k \quad [2]$$

Thus, the value for P is determined by the respective values for S and k . Integration of equation [1] gives

$$P = \left(1 - \frac{S}{k} \right) e^{-kt} + \frac{S}{k} \quad [3]$$

This highly simplified model is presented only to facilitate an understanding of some of the processes involved in changing enzyme levels in animal tissues. Multiple factors, largely unknown, are obviously involved in controlling the rates of both enzyme synthesis and enzyme degradation (19).

evidence that there is a continual degradation of the enzyme under basal conditions; *i.e.*, the enzyme is continually turning over. In contrast, when tryptophan was administered to the animals, there was no decrease in the amount of prelabeled enzyme. Thus, the substrate (tryptophan) allowed for an increase in the enzyme level by preventing degradation of the active enzyme in the presence of its continued synthesis.

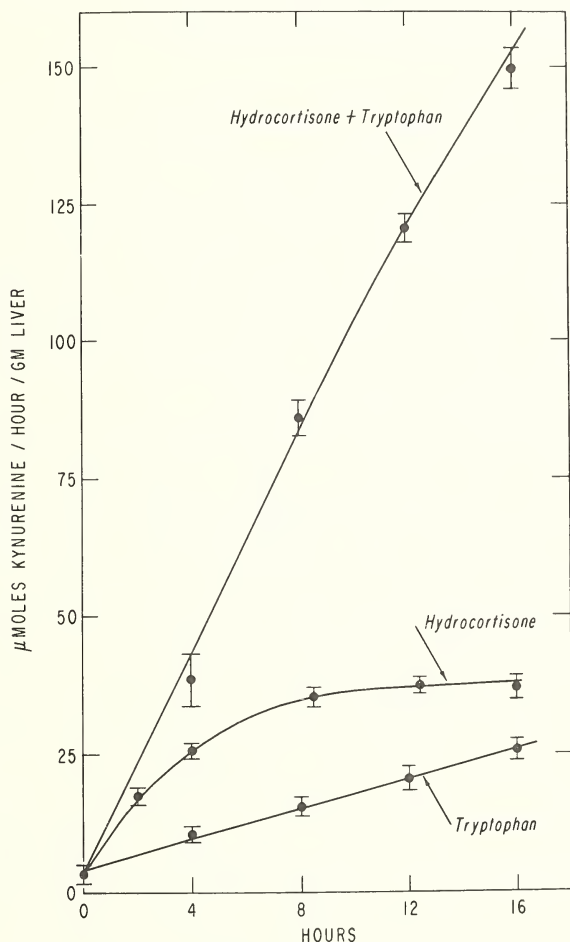


TEXT-FIGURE 3.—Effect of L-tryptophan administration on the loss of tryptophan pyrrolase prelabeled with ^{14}C -L-leucine. Rats were given single injections of 20 μC of ^{14}C -L-leucine. Sixty minutes later, two animals were killed. The remainder were given 10 ml of 0.85% NaCl or 10 ml of 0.85% NaCl containing 150 mg of L-tryptophan. These injections were repeated in the remaining animals after 4 and 8 hours. At the times specified, the livers of two animals in each group were removed and frozen immediately. At the end of the experiment, extracts of the livers were prepared, and the radioactivity that was incorporated into tryptophan pyrrolase and protein was determined. The values given are for totals of combined extracts of two animals; (●—●) enzyme activity; (▲—▲) total radioactivity in protein precipitated by the tryptophan pyrrolase antiserum; (■—■) radioactivity in total cellular protein.

The interactions between altered rates of synthesis and degradation can be seen in text-figure 4, which shows the time course of increases in tryptophan pyrrolase following administrations of either tryptophan or hydrocortisone, or both, to adrenalectomized rats at 4-hour intervals. Tryptophan results in a nearly linear increase in enzyme amounting to fivefold in 16 hours. Hydrocortisone results in an initial, rapid accumulation of enzyme, followed by a plateau after about 6 hours. The administration of both results in a near linear increase

to levels 25-fold to 50-fold greater than basal levels. These results can be explained on the basis of hydrocortisone increasing the rate of enzyme synthesis about fourfold to sixfold without affecting the rate of enzyme degradation, whereas tryptophan administration does not alter the rate of enzyme synthesis, but diminishes the rate at which the enzyme is inactivated or degraded (*see* footnote 4, p. 307).

The activity of this one enzyme, tryptophan pyrrolase, then, is controlled by multiple factors: 1) activation of the apoenzyme by com-



TEXT-FIGURE 4.—Time course of increases in tryptophan pyrrolase activity produced by repeated administrations of hydrocortisone and tryptophan. Adrenalectomized rats were given injections as follows every 4 hours: 150 mg of L-tryptophan in 12 ml of 0.85% NaCl intraperitoneally and 5 mg of hydrocortisone subcutaneously. Brackets indicate ± 2 standard errors of the mean of 4 animals in each group.

bination with hematin (22) and 2) accumulation of enzyme as a result of stimulation of the rate of enzyme synthesis, as produced by hormone, and 3) accumulation of enzyme as a result of substrate stabilization in the presence of continued enzyme synthesis. Any one of these mechanisms, then, may be operational, depending on the immediate demands. Hence, the presence of a small amount of tryptophan might involve only the activation phenomenon, whereas in the presence of a continual supply of tryptophan, stabilization of enzyme would occur. An increased rate of enzyme synthesis might become operative only under more stressful conditions in which a rapid increase in enzyme content would be required.

ON THE MECHANISM OF PROTEIN DEGRADATION

It is known that intracellular protein is generally replaced, and that it is eventually degraded to the level of amino acids. Information about how this occurs and how it is controlled, however, is rudimentary. For instance, in the above-cited case of tryptophan pyrrolase one may ask: 1) What is the mechanism for the stabilization of tryptophan pyrrolase *in vivo* by the administration of tryptophan, and 2) what is the nature of the process *in vivo* for the inactivation and degradation of intracellular proteins?

Purified tryptophan pyrrolase is unstable in the absence of tryptophan, and like many enzymes *in vitro*, is stabilized by its substrate against a variety of inactivating agents, including heat, urea, organic solvents, and proteolysis (25). Therefore, as a first approximation it would seem most reasonable to propose that tryptophan stabilization *in vivo* is similar to the *in vitro* state and the stabilization results from a direct effect of altering the protein conformation such that the enzyme is no longer subject to action of intracellular proteases, or other inactivating enzymes, perhaps those associated with lysosomes.

There are several major difficulties in reaching any definitive conclusion as to the mode of action of an agent in protecting against the physiological loss of an enzyme that occurs *in vivo*: 1) an inability to identify the products of the inactivation or degradation process once the enzyme has been sufficiently altered to irreversibly lose its catalytic or immunologic competency. This could theoretically involve only a change in the conformation or aggregational state of the enzyme rather than a splitting of peptide bonds; 2) our lack of knowledge of the enzymatic processes involved in the intracellular degradation of protein. Available evidence would suggest that this process may be highly complex. For instance, the inactivation (or degradation) of tryptophan pyrrolase, as measured by the double criteria of loss of enzyme activity and immunologic reactivity, can be

demonstrated only in structurally and metabolically intact tissues (25).

The sensitivity of the inactivation process to metabolic inhibitors can be seen from the following experiments summarized in table 2. In these experiments the levels of tyrosine transaminase and tryptophan pyrrolase were increased to high levels by the administration of hydrocortisone and tryptophan 8 hours prior to the time indicated as zero. At zero time the listed treatments were instituted, and their effect on the normally occurring decrease in enzyme activity determined 5 hours later. In intact animals that received only saline at zero time, tyrosine transaminase and tryptophan pyrrolase activities decayed to 23% and 36% of zero time values, respectively. When the livers were removed and incubated at 37 C for 5 hours, there was essentially no loss of either enzyme activity. The administration of L-tryptophan completely abolished the loss of tryptophan pyrrolase, and significantly reduced the decay of tyrosine transaminase. Most striking was the finding that inhibitors of protein synthesis, puromycin and cyclohexamide, completely abolished the decay in tyrosine transaminase, but had essentially no effect on tryptophan pyrrolase. In addition, actinomycin D significantly diminished the decay of both enzymes. In the case of tryptophan pyrrolase, it could be further shown that this effect of actinomycin D was not related to an effect in increasing enzyme synthesis (28), since the concomitant administration of puromycin did not abolish the actinomycin D effect in decreasing enzyme inactivation.

TABLE 2.—Effects of various agents on the decay of tryptophan pyrrolase and tyrosine transaminase in rat liver*

Treatment	% of initial activity†	
	Tryptophan pyrrolase	Tyrosine transaminase
Saline (control).....	36	23
Tissue death at 37 C‡.....	104§	85§
L-Tryptophan 	114§	51§
Puromycin¶.....	41	112§
Cyclohexamide**.....	40	109§
Actinomycin D††.....	59§	51§
Actinomycin D + puromycin.....	62§	—

*Male, adrenalectomized rats were given (intraperitoneally) 2 mg hydrocortisone and 100 mg/100 g body weight L-tryptophan. Eight hours later, animals received the various agents listed. Some animals were killed at that time, and all others were killed 5 hours later, and their livers assayed for tyrosine transaminase (26) and tryptophan pyrrolase (27). Results are expressed as the percent of activity remaining in the liver 5 hours after treatment compared to that initially present at the start of such treatment.

† Based on 3–5 animals in each group.

‡ Liver incubated intact at 37 C.

§ Significant differences from control $P > 0.05$.

|| 100 mg/100 g body weight.

¶ 7.5 mg/100 g body weight each hour.

**300 mg/100 g.

†† 200 mg/100 g initially.

These results are perhaps most important in indicating the dangers in using various agents for the evaluation of mechanisms of regulation of enzyme levels without first considering their potential effect on multiple cellular processes, however indirect that effect might be. Certain of these treatments, including death of the animal and administration of tryptophan, could be explained on the basis of the accumulation of small molecules, perhaps most specifically tryptophan, which either has a direct effect on stabilizing specific enzymes or inhibits a proteolytic enzyme(s). In the case of the striking effect of inhibiting protein synthesis on the decay of tyrosine transaminase, it is tempting to conclude that a protein is synthesized that specifically combines with the enzyme to facilitate its removal and/or degradation. Proteins capable of specific enzyme inhibition have been described in bacteria (29) and yeast (30). On the other hand, in several bacterial systems, agents which inhibit protein synthesis also inhibit protein degradation, perhaps because of an inhibition of a protease by amino acids or amino acyl-sRNA's accumulating as a result of inhibiting protein synthesis (31). Such a process may underlie the inhibition of inactivation of tyrosine transaminase.

CONCLUSION

The studies described herein have indicated that the existence of protein turnover in rat liver can have a metabolic regulatory function. It is apparent that the process of protein turnover and its control needs much clarification. The marked heterogeneity of turnover rates of various enzymes is striking. Although the biochemical basis for such heterogeneity is unknown, we might suggest a physiological role for heterogeneity. Thus, enzymes whose optimal levels of activity are rate-limiting for a specific biochemical reaction *in vivo* may have a rapid rate of turnover. Such enzymes would respond rapidly, both increasing and decreasing in response to metabolic demands. On the other hand, enzymes that are in constant usage or whose physiological activity is controlled by activation, feedback inhibition, or the availability of substrate would not be required to fluctuate rapidly, and hence would tend to have slower turnover rates.

RESUMEN

En oposición a las bacterias en crecimiento logarítmico, existe una síntesis continua y considerable, y una degradación del mismo nivel (recambio) de proteína total, así como de las enzimas específicas, en los tejidos animales. Por lo tanto, un nivel enzimático dado es la suma algebraica de ambos procesos de síntesis y degradación,

cada uno de los cuales puede alterarse independientemente por cambios en los estados fisiológico o nutricional, o por la administración de hormonas u otros compuestos.

En el caso de la triptofano pirrolasa, la administración de glucocorticoides aumenta el nivel de la enzima como resultado de un aumento en la velocidad de la síntesis de la enzima, mientras que la acumulación de la enzima inducida por el sustrato puede atribuirse a la detención de la degradación enzimática, es decir, a estabilización de la enzima, en presencia de una síntesis persistente de la enzima.

Existe una marcada heterogeneidad de las velocidades de recambio de diferentes enzimas en el hígado de rata, con vidas medias que varían desde varias horas hasta 4 a 5 días. Los niveles de las enzimas con velocidades de recambio muy rápidas serán más susceptibles y responderán mejor (cambio en la cantidad de veces que aumenta el nivel de una enzima) a la alteración de las velocidades de síntesis o de degradación, que las que tienen un recambio bajo. Así, la administración de cortisona aumenta la velocidad de la síntesis de la triptofano pirrolasa, de la glutamina-alanina transaminasa y de la arginasa, más o menos en la misma proporción. Sin embargo, el tiempo empleado en el aumento varía en cada caso debido a las diferentes velocidades de recambio para cada enzima.

El o los mecanismos y la regulación de los sucesos que participan en la degradación de las enzimas no se conoce bien por el momento. Los agentes o las drogas que inhiben el metabolismo energético y la síntesis de proteínas. Por lo tanto, el proceso de la degradación de proteínas es un proceso complejo que es alterado por numerosos factores.

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Hormonal Regulation of Liver Enzyme Synthesis^{1,2,3}

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SUMMARY

Induction of hepatic tyrosine- α -ketoglutarate transaminase by hydrocortisone has long served as a model system for the study of the mechanism by which hormones regulate the synthesis of specific enzyme proteins. The steroid stimulates synthesis of all classes of liver RNA *in vivo*, a result which is difficult to reconcile with the specificity of the hormonal stimulation of enzyme synthesis. In a search for extra-adrenal factors which might be operating to regulate synthesis of this enzyme, we have identified three protein hormones which are capable of altering the rate of transaminase synthesis *in vivo*. The hypophyseal growth hormone brings about an essentially complete, selective repres-

sion of transaminase synthesis. When repression occurs, the enzyme level falls at the rate expected from the known turnover rate of this enzyme. Both of the pancreatic hormones, insulin and glucagon, increase the rate of transaminase synthesis and thereby elevate the enzyme level threefold to fourfold within a few hours. The protein hormones, like the steroid hydrocortisone, have no effect on enzyme synthesis if RNA formation is blocked by actinomycin. It is postulated that the various hormonal regulators may all act to regulate the synthesis of a repressor which governs transaminase synthesis at the translational level.—*Nat Cancer Inst Monogr* 27: 315-323, 1967.

INDUCTION OF tyrosine- α -ketoglutarate transaminase and of other enzymes of rat liver by adrenal glucocorticoid hormones has been studied intensively in recent years. These studies have been carried

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² See Discussion of this paper conducted by Dr. G. D. Novelli, p. 325.

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out in the enormously complex environment of the living animal, with the attendant possibility of confusion of cause and effect due to the participation of unrecognized factors operating *in vivo*. In the present series of experiments, we have identified three nonadrenal hormones which also regulate the synthesis of hepatic tyrosine transaminase. Each of these can act independently of the adrenal hormones and of each other, and it is thus apparent that the regulation of enzyme synthesis in the intact animal is, indeed, more complex than was hitherto supposed. Details of the experiments described here can be found in the original publication (1).

REPRESSION BY GROWTH HORMONE

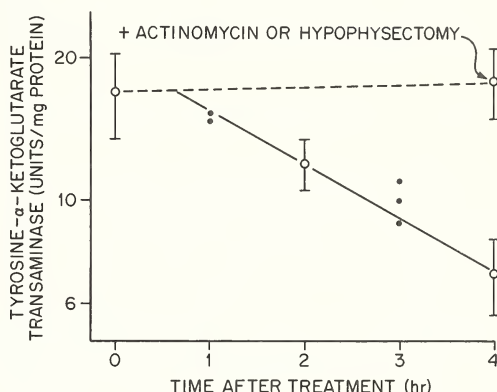
In previous experiments designed to analyze the role of the substrate, tyrosine, in the regulation of tyrosine transaminase synthesis, it was found that the amounts usually administered (2) of this amino acid were actually exerting a profound stressing effect on the pituitary-adrenal system, and thereby effecting a glucocorticoid induction in animals with intact adrenals (3). However, the transaminase level was markedly *lowered* when adrenalectomized rats were given intraperitoneal injections of tyrosine or of the insoluble diatomaceous earth, Celite (3). An examination of the time course of this response yielded the data presented as a semilog plot in text-figure 1. After an initial response which appears as a lag in text-figure 1, but which was later found to be quite variable (*see below*), the transaminase level fell in first-order fashion, with a half-life of about 2.5 hours. As discussed by Segal and Kim (4) and by Schimke *et al.* (5), the kinetic parameters governing the level of enzyme include a zero-order rate of synthesis and a first-order rate of degradation. Previous estimates of the half-life of tyrosine transaminase degradation *in vivo* (6-8) range from 2-4 hours, and thus the rate of the fall in tyrosine transaminase which follows stress is consistent with the conclusion that synthesis of this enzyme has been repressed, probably completely.

Actinomycin blocks the response to stress; the transaminase level is unchanged in stressed animals pretreated with this inhibitor of DNA-directed RNA synthesis (text-fig. 1). This result implicates transcriptional events in the response to stress, but also indicates that the inhibition of enzyme synthesis occurs at the level of translation, that is, the RNA-directed assembly of amino acids into the enzyme protein. This follows from the fact that actinomycin has no effect on enzyme synthesis, which suggests that the RNA template for synthesis of this enzyme is stable, at least for the period of these experiments (4 hours). If the template is stable, then repression of synthesis must be translational. This argument was first advanced by Garren *et al.*

(9) to explain the paradoxical effect of actinomycin given several hours after an inducing dose of hydrocortisone.

As might be expected for a stress-initiated response, transaminase repression does not occur in hypophysectomized rats treated with stressing agents (text-fig. 1).

TEXT-FIGURE 1.—Repression in stressed adrenalectomized rats. The animals were given intraperitoneal injections at zero time of either tyrosine (*open circles*) or Celite (*closed circles*), 60 mg/100 g. Vertical bars represent standard errors for 6 to 8 animals for each point. Actinomycin (100 μ g/100 g) was given shortly before the stressing agent. Adapted from Kenney and Albritton (1).



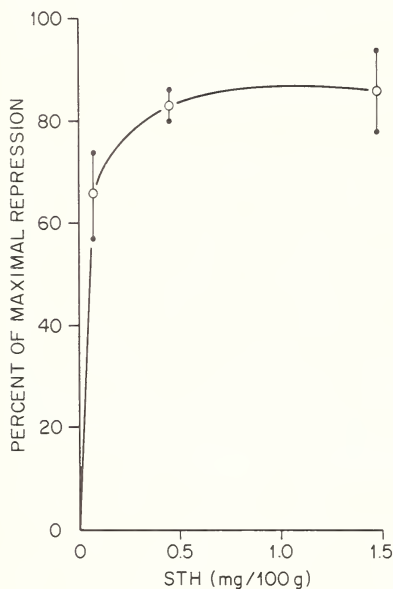
A further indication of the involvement of pituitary hormones was obtained in parabiotic experiments. Here it was shown that repression does occur in hypophysectomized rats joined parabiotically to stressed animals bearing a functional pituitary (text-fig. 2).

TEXT-FIGURE 2.—Repression in parabiotic rats. Hypophysectomized rats were parabiotically joined to normals 10–14 days, and all the rats were adrenalectomized 2 days before the experiments were done. Stressing was accomplished by intraperitoneal injections of tyrosine. The data are hepatic tyrosine transaminase (units per mg protein; mean \pm maximal variation) 4 hours after the stressing agent was given. Number of observations in parentheses.

TREATMENT	ENDOCRINE STATUS	
	A HYPOX, ADRX	B ADRX
NONE (3)	24 \pm 4	21 \pm 2
STRESS $\underline{\text{B}}$ (4)	13 \pm 2	12 \pm 3
STRESS $\underline{\text{A}}$ (1)	20	26

Subsequent analyses eventually revealed that the hypophyseal growth hormone (somatotropin, STH) is the pituitary component active in bringing about repression of transaminase synthesis in the liver. As shown by the dose-response curve (text-fig. 3), the dose required is not large, a marked repression being observed with a dose

(60 $\mu\text{g}/100\text{ g}$) similar to that employed in growth-promoting analyses. Since the half-life of this enzyme is now known with some accuracy ($t_{1/2} = 1.3\text{--}1.5$ hours), we can estimate the enzyme level expected in a given experiment if repression begins immediately and is complete. As shown in text-figure 3, the maximal repression observed is 80–90% of this theoretical limit. Thus the repression response is either not initiated immediately after growth hormone treatment, or the repression of enzyme synthesis is not completely effective.



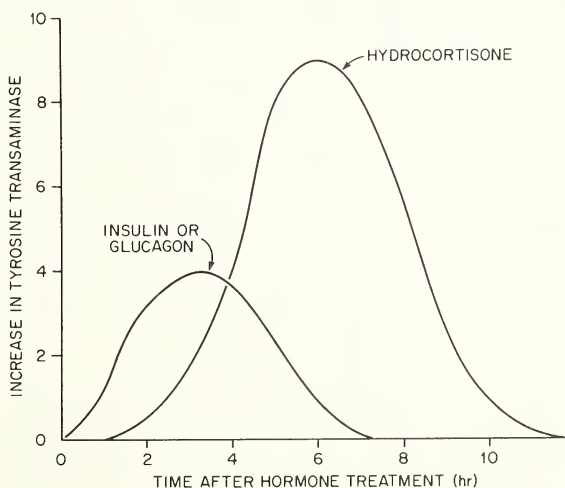
TEXT-FIGURE 3.—Repression by growth hormone in hypophysectomized rats. Tyrosine transaminase was measured 4 hours after administration of the dose indicated of sheep growth hormone (NIH:GH:S7).

That the first of these alternatives represents the correct explanation was determined in studies of the time course of the repression response. In these experiments, it was found that the initial response of hepatic tyrosine transaminase to the administration of growth hormone was extremely variable. In some cases, the transaminase level was already lowered after 1 hour, and continued to fall in first-order fashion for several hours thereafter, as in the response to stress depicted in text-figure 1. In other experiments, the enzyme level rose during the first 1 or 2 hours after hormone treatment, reaching a peak 50 to 100% higher than the original level. Still other experiments resulted in an extended lag period of 1–2 hours during which the enzyme level remained essentially constant. In each case, the variable initial phase was followed by a phase in which the transaminase level fell in the usual first-order fashion. This variable response was found in adrenalectomized rats subjected to stress as well as in growth

hormone-treated rats that were either adrenalectomized or hypophysectomized or both. We concluded that some capacity for transaminase induction probably remains in rats deprived of glucocorticoids. Our results could then be explained if growth hormone treatment resulted in stimulation of this inducing capacity which, being variable in response, might be due to the action of other hormones released in animals treated with growth hormone.

INDUCTION BY PANCREATIC HORMONES

Insulin release in response to growth hormone has been demonstrated (10). A limited induction of tryptophan pyrrolase in adrenalectomized rats given large doses of insulin was shown by Schor and Frieden (11). We found that synthesis of tyrosine transaminase is stimulated by small doses of glucagon-free insulin in rats that were either adrenalectomized or hypophysectomized or both. Larger quantities of glucagon cause an essentially identical response. The amounts required make it most unlikely that the effect of either of these hormones can be attributed to contamination with the other, and each is effective in induction experiments *in vitro* with hepatoma cells in tissue culture. The time course and extent of induction with these protein hormones differ from the induction by hydrocortisone, as illustrated in text-figure 4. Response to either of the protein hormones is apparent earlier than that to hydrocortisone. The maximal induction obtained is limited (threefold to fivefold), and the enzyme level begins



TEXT-FIGURE 4.—Induction in adrenalectomized rats.

to return to normal after about 3 hours, while the steroid effect continues for 6–7 hours. The extent to which these differences may reflect different rates of entry and catabolism of the protein hormones, in contrast to those of the steroid, is not yet known.

Evidence that the changes in enzyme levels shown earlier actually reflect changes in the rate of transaminase synthesis is summarized in table 1. In these experiments, the enzyme is labeled in a brief "pulse" exposure to ^{14}C -amino acids; if the duration of labeling is short relative to the turnover time of the enzyme, the extent of ^{14}C -amino acid incorporation into the enzyme is a direct estimate of its rate of synthesis (4, 5, 12). The enzyme is isolated by precipitation with anti-transaminase serum (13). Individual variation in the extent of dilution of the administered isotopic amino acid is corrected for by expressing transaminase radioactivity relative to that of the total soluble liver proteins. Growth hormone effected a decrease in the rate of transaminase synthesis to about 14% of the control rate, while hydrocortisone and the pancreatic hormones stimulated synthesis threefold to sevenfold. These experiments provide only estimates of the magnitude of the hormonal effects on enzyme synthesis, since we have recently recognized that the labeling interval was too long in some experiments and that the duration of hormone treatment was poorly chosen in others. Nevertheless, it is clear that the hormones bring about changes in the transaminase level by stimulating synthesis (induction by hydrocortisone, insulin, or glucagon) or by inhibiting synthesis (repression by growth hormone).

The relationships involved in the neuroendocrine control of synthesis of tyrosine transaminase are depicted in text-figure 5, in which

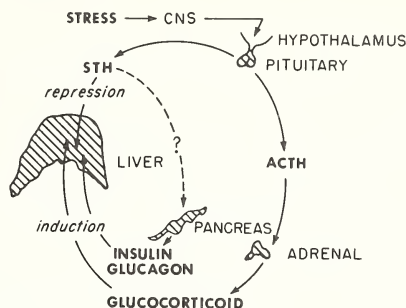
TABLE 1.—Immunochemical analyses of hormonal effects on transaminase synthesis*

Experiment No.	Hormone treatment	Labeling		Transaminase units/mg protein	Relative specific radioactivity
		Time (hr)	Interval (min)		
1	None	—	20	41	1.0
	Growth hormone	4	20	19	0.14
2	None	—	40	13	1.0
	Hydrocortisone	4	40	117	7.0
3	None	—	10	38	1.0
	Glucagon	1.5	10	91	6.2
4	None	—	20	31	1.0
	Insulin	2.5	20	114	2.9

*Adrenalectomized rats were treated with the following doses of the respective hormone, expressed per 100 g of body weight: insulin, 0.8 units (35 μg); glucagon, 100 μg ; hydrocortisone, 2 mg; growth hormone, 1.3 mg. At the indicated time after hormone treatment, ^{14}C -amino acids (20 to 50 μc , the amount being constant in each experiment) were given, and after the indicated interval the animals were killed and their livers removed for assay. Transaminase radioactivity is expressed relative to that of the total soluble proteins of the liver and has been normalized to a control level of 1.0 in each experiment. The data are averages for 3 or 4 animals in each case.

the dashed line reflects our uncertainty that the variation in response to growth hormone actually is due to pancreatic stimulation and a resultant induction by insulin. The other relationships shown are well established.

TEXT-FIGURE 5.—Neuroendocrine relationships in control of hepatic enzyme synthesis.



The complexity of the *in vivo* experimental system for the study of regulatory mechanisms is readily apparent, which points up the significance of the recent development of cultured cell lines capable of responding to hormonal control of enzyme synthesis (14, 15). Nevertheless, it has been possible to discern some features of the cellular mechanisms upon which these hormones operate. All of the hormone effects described here are blocked by actinomycin. This result shows that transcriptional processes must be intact for the hormones to exert their effects on enzyme synthesis, and suggests that the hormones may act, directly or indirectly, to alter genetic transcription in a specific fashion. Hepatic RNA synthesis is, in fact, increased by hydrocortisone (16, 17), insulin (18), and growth hormone (19, 20), but the anticipated specificity of these effects has not yet been convincingly demonstrated. While these considerations suggest that the primary action of all of these hormones is upon transcriptional events, the available evidence (discussed earlier) indicates that the repression of enzyme synthesis promoted by growth hormone involves a shutdown of translation. Thus it might be supposed that growth hormone alters genetic transcription, resulting in the formation of a specific repressor of transaminase synthesis, which acts at the ribosomal level.

That the protein hormones from the pancreas and the adrenal steroid hormones are able to effect the same induction process introduces a new complexity into our considerations on mechanisms. We do not yet have any evidence to permit a conclusion on the important question of whether all these hormones act in the same or in different fashions to effect transaminase induction. But clearly the razor of Occam is best satisfied if we assume that some essential feature of the induction process is similarly influenced by all three hormones. Synthesis of the

hypothetical repressor presents an obvious choice for this control point, for if it were inhibited by these hormones (perhaps in quite different fashion), each would bring about transaminase induction.

RESUMEN

La inducción de transaminasa tirosina- α -cetoglutarato hepática por la hidrocortisona ha servido, desde hace mucho tiempo, como modelo para el estudio del mecanismo por medio del cual las hormonas regulan la síntesis de la proteínas enzimáticas específicas. El esteroide estimula la síntesis de toda clase de ARN hepático *in vivo*, resultado que es difícil de reconciliar con la especificidad de la estimulación hormonal de la síntesis de enzimas. En una búsqueda de los factores extra-adrenales que pueran participar en la regulación de la síntesis de dicha enzima, hemos identificado tres hormonas proteínicas que pueden alterar, *in vivo*, la velocidad de la síntesis de la transaminasa. La hormona de crecimiento de la hipófisis produce una represión selectiva, prácticamente completa, de la síntesis de la transaminasa. Cuando ocurre la represión, el nivel de la enzima cae a la velocidad esperada en vista de la velocidad de recambio conocida de esta enzima. Las dos hormonas pancreáticas, insulina y glucagon, aumentan la velocidad de síntesis de transaminasa y, por lo tanto, suben el nivel de la enzima 3 a 4 veces en el término de unas horas. Las hormonas proteicas, como el esteroide hidrocortisona, no tienen efecto sobre la síntesis de enzima si la formación de ARN se bloquea con actinomicina. Se postula que diversos reguladores hormonales pueden actuar en conjunto para modificar la síntesis de un represor que gobierna a la síntesis de transaminasa, al nivel de la traducción.

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DISCUSSION ¹

*Discussor, DR. G. DAVID NOVELLI, Biology Division,
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I WOULD like to congratulate Drs. Schimke, Kenney, and Tomkins for very lucid presentations of a very complex situation. I am sure that all of us were impressed by the complexity of the regulation of protein synthesis in mammalian liver. The concept that the level of activity of an enzyme in the liver at any one time is a consequence of the rate of its synthesis as well as the rate of its degradation introduces an added component in regulation. Changes in the rate of synthesis or the rate of degradation might be differently affected by different hormones. The ideas presented in these papers certainly suggest that some, and perhaps a great deal, of the regulation of protein synthesis takes place at the translational level in mammalian liver. I have no specific questions for these three speakers. However, I would like to suggest another possible regulatory mechanism that might apply to this particular situation and that is specific changes in transfer RNA. According to our current concept of the genetic code, there are 64 or 62 codons (leaving out Amber and Ocher) that can be used to specify the amino acids in proteins. In a simple view, having 62 codons could mean that a cell has 62 different transfer RNAs—for example we know that leucine has 6 codons, and 5 leucine tRNAs (iso-accepting) have been found in *E. coli*. It seems unlikely that a cell would be using all 62 codons in its messenger RNA at any one particular moment. Since the iso-accepting tRNAs are not present in equal amounts, it might be that the rate of synthesis of a particular protein is limited because its mRNA contains the code for one of the minor components of an iso-accepting set of tRNAs. In such a case the rate of synthesis of that particular protein could be changed by changing the amount of that species of tRNA. Such a change in a single component of tRNA would go undetected with our present methods of analysis for the synthesis of RNA.

¹ Of articles by Gordon M. Tomkins and Bruce N. Ames; Robert T. Schimke; and Francis T. Kenney, Darold Holten, and William L. Albritton.

General discussion was opened by Dr. Hermann Niemeyer who spoke to the point raised in Dr. Schimke's talk that enzyme induction in mammalian liver differs from induction in bacterial system in that the basal level of enzyme in the liver is generally much higher than in an uninduced bacteria cell. Niemeyer questioned whether the basal level of enzyme could be isoenzymes and that the enzyme induced after cortisone was a different form that was not present prior to induction. Dr. Schimke replied that for tryptophan pyrrolase, the basal enzyme chromatographs at the same ionic strength as the induced enzyme, and Dr. Kenney pointed out he has seen no evidence of isoenzymes of the tyrosine transaminase and that the induced enzyme is identical to the basal enzyme in all respects that have ever been examined. Dr. Lardy added that in the case of phosphoenopyruvate carboxykinase both chromatographic evidence and sucrose density gradient centrifugations indicate that the induced and the original enzyme are identical. In commenting about the high basal level of the liver enzyme, Schimke pointed out that even in basal conditions there is always a certain amount of other hormones that may be involved in enzyme synthesis. If one could be completely free of hormones, the basal level could be quite low. Schimke said that this is what happens with liver cells in culture, that specific liver enzymes are lost and suggested that perhaps the absence of hormones might be responsible for their loss. Niemeyer called attention to the basic difference in the kinetics of induction of tryptophan pyrrolase as described by Schimke and that of glucokinase described earlier by Niemeyer. The increase and decrease of tryptophan pyrrolase show it to be a very symmetric process, whereas glucokinase reaches its maximum in 6 hours and the decline requires 33 hours. This would appear to be in conflict with the finding of Schimke that the rate of synthesis of the liver enzymes following induction by cortisone is determined by the rate of decay of the enzyme. Schimke replied that the model described, where there is a zero-order rate for synthesis and a first-order rate for the decay of the enzyme, is highly simplified and to do real justice to the kinetics would require the addition of a number of other parameters whose values are not presently available.

Dr. Philip Cohen reported on the effect of thyroxine on the synthesis of carbamyl phosphate synthetase in liver of tadpoles. Administration of thyroxine to tadpoles induces precocious metamorphosis and stimulates the *de novo* synthesis of carbamyl phosphate synthetase in the liver. In discussing recent experiments from his laboratory, Dr. Cohen said that there is reasonably good evidence that thyroxine has an effect at both the transcriptional and translational levels and of the same order of magnitude. He cited the observation that chromatin prepared from thyroxine-treated animals is about three times as efficient as a template for RNA synthesis as is chromatin from un-

treated animals. At the same time, the capacity to incorporate amino acids into protein is increased to the same level.

Dr. Lardy questioned Dr. Kenney regarding the observation that two such antagonistic hormones as glucagon and insulin have similar effects on the synthesis of tyrosine transaminase. He recalled the fact that administration of insulin results in the release of glucagon and administration of glucagon causes the release of insulin in the rat. He asked whether these hormones had been tested in the diabetic animal. Dr. Kenney replied that he believes the two hormones act independently, since each gave the same effect when added to cells in culture.

Dr. Koshland suggested that perhaps the total mRNA synthesis in cells is constant and that to increase the synthesis of one enzyme would require the selection of increased synthesis of this mRNA at the expense of a decreased synthesis of mRNA's for other enzymes. Dr. Tomkins replied that this was a reasonable suggestion. He cited the observations of Harris, Shearer, and McCarthy that a large amount of RNA turns over rapidly in the nucleus of mammalian cells but does not appear in the cytoplasm. He estimated that about 20% of the genome is transcribed but only about 6% of the RNA appears in the cytoplasm, so that the other 14% is just degraded. He suggested that only those messengers that can be translated are selected to appear in the cytoplasm. The discussion ended with a comment by Dr. Gaede who questioned whether the effects of the hormones discussed were primary or secondary effects and reflected that the answer to this question would be difficult to find.

CLOSING LECTURE

Chairman: Alexander Hollaender

Role of the Redox State of Nicotinamide Adenine Dinucleotides in the Regulation of Metabolic Processes^{1,2}

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SUMMARY

The concentrations of the oxidized and reduced substrates of the lactate, β -hydroxybutyrate, and glutamate dehydrogenase systems were measured in rat liver that had been freeze-clamped as soon as possible after death. The substrates of these dehydrogenases are in near equilibrium with free NAD and NADH₂, and the ratio of the concentrations of the free dinucleotides can therefore be calculated from the measured concentrations of the substrates and the equilibrium constants. The lactate dehydrogenase system reflects the NAD/NADH₂ ratio in the cytoplasm, whereas the β -hydroxybutyrate dehydrogenase and the glutamate dehydrogenase systems reflect this ratio in the mitochondrial cristae and matrix, respectively. The mean NAD/NADH₂ ratio, at pH 7.0, of rat liver cytoplasm was calculated to be 725 in well-fed rats, 528 in starved rats, and 208 in alloxan diabetic rats. The NAD/NADH₂ ratios for the mitochondrial matrix and cristae gave virtually identical values in the same metabolic state. This indicates that

β -hydroxybutyrate and glutamate dehydrogenases share a common pool of dinucleotide. The mean NAD/NADH₂ ratio within the mitochondria of well-fed rat liver was about 8. It fell to about 5 in starvation and rose to about 10 in alloxan diabetes. The NAD/NADH₂ ratios of cytoplasm and mitochondria are thus greatly different and do not necessarily move in parallel when the metabolic state of the liver changes. The bearing of these findings on various problems, including the following, is discussed: the number of NAD-NADH₂ pools in liver cells; the applicability of the method used to tissues other than rat liver; the physiological significance of the differences of the redox states of mitochondria and cytoplasm; aspects of the regulation of the redox state in cell compartments; and the relations between the redox state of cell compartments and ketosis. Provisional data on the redox state of the NADP couple in rat liver are given. —*Nat Cancer Inst Monogr* 27: 331–343, 1967.

¹ Presented at the International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico, November 28 to December 1, 1966.

² For a full account *see* (1).

THE REDOX STATE of the nicotinamide adenine dinucleotide couple is characterized by the value of the ratio

$$\frac{\text{concentration of free NAD}}{\text{concentration of free NADH}_2}$$

(hereafter referred to as the NAD/NADH₂ ratio). "Free" in this term is meant to exclude protein-bound nucleotides. Information on the value of this ratio at the site of the oxido-reductions is of interest because it bears on the metabolic behavior of oxidizable and reducible substrates. This ratio is especially relevant to two aspects of cell metabolism: 1) it determines the direction of reversible reactions, *e.g.*, whether the triose-phosphate dehydrogenase reaction occurs in the direction of glycolysis or of gluconeogenesis; and 2) it determines the magnitude of the free-energy changes of oxido-reductions, *e.g.*, those accompanying the transfer of electrons from NADH₂ to flavo-proteins in the electron transport chain. Unless the free-energy change of this reaction is above a critical minimum, the electron transfer cannot be effectively coupled with the synthesis of ATP.

The value of the NAD/NADH₂ ratio cannot be obtained by direct measurements of the tissue content of NAD and NADH₂. Such measurements fail to differentiate between the free and protein-bound nucleotides, and they give no information on the distribution of the nucleotides between the various cell compartments, which is known to be uneven. The latter difficulty cannot be dealt with by the usual methods of tissue fractionation because the distribution of the nucleotides is liable to undergo very rapid changes during the process of fractionation. Both kinds of difficulty—the distinction between free and bound nucleotides and the uneven distribution within the cell—may be overcome by measuring the ratio concentrations of the oxidized and reduced metabolites of suitable NAD-linked dehydrogenase systems which, on account of their high activity, are practically in equilibrium with the nucleotides, according to the equation

$$\frac{[\text{oxidized substrates}][\text{NADH}_2]}{[\text{reduced substrates}][\text{NAD}]} = K. \quad [1]$$

If K is known, the NAD/NADH₂ ratio can be calculated from the concentrations of the substrates. K , it should be noted, is the equilibrium constant divided by the H⁺ concentration. For the purpose of the calculations in this paper, H⁺ of the liver was taken to be 10⁻⁷.

These principles were first clearly stated by Holzer and Lynen (2, 3) and by Bücher and Klingenberg (4). An important feature of the principle is the fact that equation [1] can supply the NAD/NADH₂ ratio for the cell compartments in which the dehydrogenase is located, although the analyses are carried out on the whole tissue. This holds if the distribution of the oxidized and reduced substrates throughout the cell is even.

CHOICE OF DEHYDROGENASE SYSTEMS

Only a small number of dehydrogenase systems are suitable for assaying the NAD/NADH₂ ratio. Hohorst *et al.* (5) showed there are three NAD-linked dehydrogenases in rat liver—lactate, α -glycerophosphate, and malate dehydrogenases—that, under certain conditions, give the same value for the NAD/NADH₂ ratio calculated according to equation [1]. This demonstrates convincingly that the dehydrogenases can establish equilibria between their substrates and the free nucleotides in the cytoplasm. Of these three systems, the lactate dehydrogenase system is the most satisfactory for general use because the activity of the α -glycerophosphate dehydrogenase is not as high as that of lactate dehydrogenase and because malate dehydrogenase is not exclusively located in the cytoplasm.

A system suitable for the assessment of the NAD/NADH₂ ratio in the mitochondria of rat liver, but not necessarily of other cells, is the β -hydroxybutyrate-acetoacetate system. The dehydrogenase is insoluble and located exclusively in the mitochondrial cristae. Most other mitochondrial NAD-linked dehydrogenase systems are unsuitable for the assay of the NAD/NADH₂ ratio, some because their activity is too low to establish equilibrium and others because there are analytical obstacles in the determination of reactants. A system satisfactory in rat liver is glutamate dehydrogenase, which is of high activity and located exclusively in the mitochondrial matrix.

On the basis of these considerations, the concentrations of the substrates of the lactate, β -hydroxybutyrate, and glutamate dehydrogenase systems were measured in the livers of rats as quickly as possible after death (6). The livers were deep-cooled within a few seconds with aluminum blocks (7). The results obtained therefore reflect *in vivo* conditions.

OBSERVATIONS

NAD/NADH₂ Ratios in Well-Fed, Starved, and Diabetic Liver

The concentrations of the substrates of the three chosen dehydrogenase systems were measured in rat liver under three different conditions—well-fed, starved (48 hours), and alloxan diabetes (table 1). Some of the differences between these three conditions were as expected. Thus, the concentration of total ketone bodies rose 10-fold in starvation and over 50-fold in diabetes above the concentration in the well-fed liver. A striking change in the diabetic liver was a major fall in the concentrations of glutamate and α -oxoglutarate and a rise in the concentration of ammonia.

METABOLIC REGULATION

TABLE 1.—Concentrations of the substrates of NAD-linked dehydrogenase systems in livers of well-fed, starved, and alloxan diabetic rats*

State of animals	Lactate	Pyruvate	Gluta- mate	α -Oxo- glutarate	NH_4^+	β -Hydroxy- butyrate	Aceto- acetate	[Lactate] [Pyruvate]	[Glutamate] [α -Oxoglutarate] $[\text{NH}_4^+]$		[β -Hydroxybutyrate] [Acetoacetate]
Well-fed	1.62	0.130	2.41	0.145	0.47	0.144	0.055	12.5	35		2.6
Starved (48 hours)	0.78	0.047	2.64	0.086	0.56	1.79	0.50	17	55		3.6
Alloxan diabetes.	2.37	0.054	0.96	0.045	0.86	7.73	3.75	44	25		2.1

*The blood sugar value of the diabetic rats was above 30 mm. The concentrations of the metabolites are expressed as $\mu\text{mole/g}$ fresh weight. For experimental details and statistical data see (6).

The lactate/pyruvate ratio rose slightly in starvation, and considerably (3.5-fold) in diabetes, as previously reported (8, 9). Unexpectedly, the changes in the mitochondrial dehydrogenase systems did not always parallel those of the cytoplasmic system. In diabetes there was a decrease in the ratios of the two mitochondrial systems, in contrast to the increase in the ratio of the cytoplasmic system. In starvation, on the other hand, the three systems moved in the same direction to approximately the same extent.

The results of the calculation of the NAD/NADH₂ ratio according to the equation

$$\frac{\text{NAD}}{\text{NADH}_2} = \frac{\text{concentration of oxidized substrate}}{\text{concentration of reduced substrate}} \times \frac{1}{K} \quad [2]$$

are shown in table 2. The values obtained for the lactate dehydrogenase system, indicating the NAD/NADH₂ ratios in the cytoplasm, essentially confirm those of previous investigators (8-10), except that the higher value for the equilibrium constant decreases the ratio to about half the value arrived at previously. The cytoplasm of the starved liver, and especially of the alloxan diabetic liver, is in a more reduced state than in the well-fed liver. The NAD/NADH₂ ratios of the mitochondria are very much lower than those for the cytoplasm, differing by a factor of about 100 in the well-fed liver and normal, starved liver and by a factor of about 20 in the diabetic liver. The most remarkable result is the finding that the glutamate and β -hydroxybutyrate systems give the same values, within the limits of error. This implies that the redox states of the mitochondrial matrix and cristae are identical. Another important result, already referred to, is the demonstration that the changes caused by alloxan diabetes in the cytoplasm and the two mitochondrial compartments are not paral-

TABLE 2.—Calculation of NAD/NADH₂ ratios in rat liver from the concentrations of the oxidants and reductants of the lactate, glutamate, and β -hydroxybutyrate dehydrogenase systems*

State of liver	NAD/NADH ₂ ratio calculated from:		
	Lactate dehydrogenase system (cytoplasm)	Glutamate dehydrogenase system (mitochondrial matrix)	β -Hydroxybutyrate dehydrogenase system (mitochondrial cristae)
Well-fed.....	725	7.3	7.8
Starved.....	528	4.7	5.6
Alloxan diabetes.....	208	10.8	9.6

*The calculations are based on the figures given in table 1 and on the following values for the equilibrium constants of the dehydrogenase systems at 38 C, pH 7.0, and an ionic strength of 0.25: lactate dehydrogenase 1.11×10^{-4} ; β -hydroxybutyrate dehydrogenase 4.93×10^{-2} ; glutamate dehydrogenase (NAD) 3.9×10^{-3} mm; pH is assumed to be 7.0.

lel. The NAD/NADH₂ ratio drops to less than one third in the cytoplasm and increases by about one third in the other compartments.

The fact that the calculations for the glutamate and β -hydroxybutyrate dehydrogenase systems lead to the same values for the NAD/NADH₂ ratios implies that the substrates of these two dehydrogenases are in equilibrium with the same NAD-NADH₂ pool. The validity of this conclusion can be checked by other calculations that do not rely on separate experimental values for the equilibrium constants. The combination of the equations for the equilibrium constants of the glutamate and β -hydroxybutyrate dehydrogenase systems shows that the following expression should be constant if the components are in equilibrium:

$$\frac{[\beta\text{-hydroxybutyrate}][\alpha\text{-oxoglutarate}][\text{NH}_4^+]}{[\text{acetoacetate}][\text{glutamate}]} \quad [3]$$

The test using the figures given in table 1 shows that this is the case:

<i>State of liver</i>	<i>Value of expression [3]</i>
Well-fed	$7.3 \times 10^{-2}\text{mM}$
Starved	$6.6 \times 10^{-2}\text{mM}$
Alloxan diabetes	$8.4 \times 10^{-2}\text{mM}$

This test involves no assumptions on the ionic strength of the tissue, on the value of the equilibrium constants, or on the *pH* of the tissue. The constancy of expression [3] may therefore be taken as proof of a joint NAD-NADH₂ pool for the two dehydrogenase systems.

The constant value obtained for expression [3] is an experimental observation. This value can also be obtained by calculation, because it is the ratio of the equilibrium constants of the glutamate and β -hydroxybutyrate systems. Using the data given in table 2, one obtains

$$\frac{\bar{K}_{\text{glutamate}}(\text{NAD})}{\bar{K}_{\beta\text{-hydroxybutyrate}}} = \frac{3.9 \times 10^{-3}}{4.93 \times 10^{-2}} = 7.9 \times 10^{-2}\text{mM}. \quad [4]$$

The agreement between this theoretical value and the observed values ($6.6 - 8.4 \times 10^{-2}\text{mM}$) is excellent.

Redox State of the NAD Couple in Liver Homogenates

If the premises of the assay principle apply to liver homogenates, then the ratio, oxidized substrate/reduced substrate, should rapidly re-establish itself to the original value after it has been disturbed by the addition of either the oxidized or reduced form of the substrate. This postulate was tested for the β -hydroxybutyrate system in rat liver homogenates. As shown in table 3 the same ratios were found within 10 minutes of the addition of either acetoacetate or β -hydroxybutyrate. This is especially striking in the experiment in which β -hydroxybutyrate was added. The addition raised the ratio by a factor of 12. Within 10 minutes the ratio changed in the opposite direction by a factor of 500 to become equal to that of the unsupplemented homogenate. The

TABLE 3.—Redox state of the β -hydroxybutyrate dehydrogenase system in rat liver homogenates*

Period of incubation (mins)	No addition			Acetoacetate (1.84 mm) added			DL- β -Hydroxybutyrate (2 mm) added		
	Concentration of:		Ratio	Concentration of:		Ratio	Concentration of:		Ratio
	Aceto- acetate (mm)	β -Hydroxy- butyrate (mm)	$\frac{[\beta\text{-Hydroxybutyrate}]}{[\text{Acetoacetate}]}$	Aceto- acetate (mm)	β -Hydroxy- butyrate (mm)	$\frac{[\beta\text{-Hydroxybutyrate}]}{[\text{Acetoacetate}]}$	Aceto- acetate (mm)	β -Hydroxy- butyrate (mm)	$\frac{[\beta\text{-Hydroxybutyrate}]}{[\text{Acetoacetate}]}$
0	0.14	0.18	1.29	1.98	0.18	—	0.07	1.13	16.1
5	0.55	0.027	0.049	2.26	0.075	0.033	—	—	—
10	0.61	0.020	0.033	2.34	0.055	0.023	1.58	0.050	0.032
20	0.64	0.025	0.039	2.33	0.082	0.035	1.72	0.058	0.033

*The homogenates were prepared in a saline medium consisting of 90 parts 1.15% KCl, 1 part 0.1 M MgCl₂, and 20 parts 0.1 M K-phosphate buffer, pH 7.4. The final concentration of the tissue was 10%. The rats were starved for 24 hours. Homogenate (4 ml) was shaken in Warburg flasks at 40 C, with O₂ in the gas space. The same homogenate was used for the unsupplemented homogenate and for the homogenate supplemented with acetoacetate. Another was used for β -hydroxybutyrate experiments.

observations confirm that the high activity of the β -hydroxybutyrate dehydrogenase keeps the concentrations of the reactants near the equilibrium state.

The experiments demonstrate another important feature of the mitochondrial redox state in homogenates. Immediately after the preparation of the homogenates, at zero time, the β -hydroxybutyrate/acetoacetate ratio was one third of that found in the starved liver *in vivo* (*cf* table 1). Within 5 minutes it fell to 1/70th of the normal value. Thus, after disintegration of the cell, the mitochondria were in a much less reduced state than *in vivo*, and this occurred after very gentle and brief treatment—homogenization of the tissue followed immediately by incubation, without centrifugal separation of the mitochondria. It is very probable that many mitochondrial experiments of previous investigators were performed on material of a highly abnormal redox state. This may have affected important characteristics, including the efficiency of oxidative phosphorylation. It seems desirable that in future work on mitochondria the redox state be defined by tests of the kind described in this section.

Addition of pyruvate and especially of succinate to the homogenate raised the β -hydroxybutyrate/acetoacetate ratio and brought it nearer the physiological range (table 4). The ready reduction of acetoacetate in the presence of succinate is a well-established phenomenon, but in previous work no measurements of the equilibrium state of the ketone bodies (*i.e.*, of the value of the NAD/NADH₂ ratio) were made.

TABLE 4.—Effects of pyruvate and succinate on the redox states of β -hydroxybutyrate and lactate dehydrogenase systems in rat liver homogenates*

Concentrations of metabolites after incubation	No pyruvate or succinate added	With pyruvate	With succinate
Acetoacetate	2.12	2.16	0.83
β -Hydroxybutyrate	0.04	0.55	1.27
Ratio $\frac{\beta\text{-hydroxybutyrate}}{\text{acetoacetate}}$	0.02	0.26	1.53
Lactate (mm)	0.65	0.87	0.71
Pyruvate (mm)	0.03	2.78	0.38
Ratio $\frac{\text{lactate}}{\text{pyruvate}}$	21.6	—	1.86

*The general conditions of incubation were as described in table 3 except that the rats were well fed. The incubation time was 10 minutes; the concentrations of added pyruvate and succinate were 10 mm and that of added acetoacetate, 2 mm.

DISCUSSION

The common NAD-NADH₂ pool of glutamate and β -hydroxybutyrate dehydrogenases in mitochondria.—Although β -hydroxybutyrate dehydrogenase is insoluble and located in the cristae—in contrast to

glutamate dehydrogenase, which is soluble and located in the matrix—a common NAD-NADH₂ pool would be expected if, as is usually the case, the dinucleotides behaved as coenzymes rather than as prosthetic groups. A coenzyme, unlike a prosthetic group, necessarily dissociates from the enzyme during each cycle of reactions and would thus not be fixed to the site of the enzyme but would be free to mix with the coenzyme molecules reacting with other dehydrogenases. The joint pool indicates that β -hydroxybutyrate dehydrogenase is located in the cristae in such a way as to combine only with the nicotinamide adenine dinucleotides of the matrix and not with those of other compartments. If the cristae and matrix share the same pool and if the “external” compartment of the mitochondria freely communicates with the cytoplasm (as is generally assumed), then there are only two major pools of free NAD-NADH₂ in the liver cell (apart from a possible third pool in the nuclei). There are, of course, large amounts of enzyme-bound nucleotides which becomes obvious when the NAD/NADH₂ ratios calculated from the equilibrium concentrations of substrates are compared with those obtained by the determination of the total dinucleotide content of the tissue. The determination of the total dinucleotides gives much lower ratios for both cytoplasm and mitochondria, differences which are due to the fact that the reduced forms of the dinucleotides are more firmly bound to the enzymes than are the oxidized forms.

Assay of the mitochondrial NAD/NADH₂ ratio.—It must be emphasized that the activities of the β -hydroxybutyrate and glutamate dehydrogenases vary greatly from tissue to tissue and are exceptionally high in rat liver. Whether the activities are high enough in other tissues to establish equilibrium requires investigation. Tests on homogenates of the kind described in this article may supply the required information.

The physiological significance of the differences of the redox state of mitochondria and cytoplasm.—The differences between the redox states of the two cell compartments are presumably essential and connected with the function of the two compartments. The cytoplasm of the liver cell is the main site of glycolysis and of gluconeogenesis, which includes the transfer of hydrogen atoms from glyceraldehyde phosphate to NAD in glycolysis, and of the reverse process in gluconeogenesis. The direction of these reactions depends on the redox state of the hydrogen carrier systems.

In the mitochondria the main function of the NAD system is to channel hydrogen atoms to the electron transport chain from the substrates of respiration. If the free-energy change of the transfer of electrons from NADH₂ to flavoprotein is to be large enough for coupling with synthesis of ATP, the NAD/NADH₂ ratio must be below a critical value.

Oxidative phosphorylation, as measured by the P/O ratio, is known to be less efficient in bacteria than in higher organisms. This may be connected with the circumstance that bacteria do not possess organelles functionally equivalent to mitochondria, *i.e.*, cell regions in which the NAD couple is in a relatively highly reduced state. Mitochondria may thus be looked upon as the outcome of an evolutionary development which *inter alia*, improved the yield of utilizable energy from the oxidative degradation of nutrients.

Redox state and diabetic ketosis.—It has been suggested (9, 11) that the redox state of the NAD-NADH₂ system in rat liver may play a role in ketogenesis by controlling the steady-state concentration of oxaloacetate. If, for one reason or another, the NAD/NADH₂ ratio fell, the ratio oxaloacetate/malate would also fall, and, if the sum of malate plus oxaloacetate remained constant, the concentrations of oxaloacetate could decrease. Lack of oxaloacetate would direct acetyl coenzyme A from the tricarboxylic cycle to ketone body formation. Experiments supporting this view have been reported by Hohorst *et al.* (8) and by Wieland and Löffler (9). However, the interpretation of the findings is not clear-cut. The analyses were carried out on the whole tissue, and unless the redox states of the various compartments moved in parallel, the data allow no conclusions on the redox state of a particular compartment. Although the present results confirm the earlier observation that the cytoplasm is more reduced in severe ketosis (the average change of the NAD/NADH₂ ratio being a decrease by a factor of 3.5), there is no parallel change in the mitochondria. On the contrary, the NAD/NADH₂ ratio in the mitochondria changed by 20–30% in the direction of oxidation. This implies that any decreases in the concentration of oxaloacetate in the mitochondria cannot be attributed to a shift of the malate/oxaloacetate ratio in favor of malate. If malate and oxaloacetate were in equilibrium in both cytoplasm and mitochondria, the malate/oxaloacetate ratio would, in severe diabetes, rise substantially in the cytoplasm and fall slightly in the mitochondria. This fall would increase the concentration of oxaloacetate at the site of the tricarboxylic acid cycle; hence, the increased formation of ketone bodies cannot be a simple consequence of the change of the redox state of mitochondria. Nevertheless, evidence discussed elsewhere (12) supports the concept that severe ketosis is caused by a fall in the oxaloacetate concentration of the liver, but that this fall is due to the rapid conversion of oxaloacetate to phosphopyruvate, and subsequently glucose.

Control of the redox state of cell compartments.—If the sum of NAD and NADH₂ is constant (*i.e.*, if the balance of the *de novo* synthesis and of the degradation of the dinucleotides is zero), the NAD/NADH₂ ratio results from the action of the dehydrogenase systems that inter-

convert NAD and NADH₂, including the NADH₂ dehydrogenases of the electron transport chain.

The limits of the NAD/NADH₂ ratio are set by the equilibrium constants of the dehydrogenase systems and by the relative concentrations of the reduced and oxidized metabolites. A survey of the main dehydrogenases of the two compartments shows that the mid-potentials of the dehydrogenases of the cytoplasm are less negative than those of most mitochondrial systems. This limits the extent of reduction of the cytoplasmic NAD much more narrowly than that of the mitochondria. Moreover, in a mixture of dehydrogenases in equilibrium with a common pool of NAD-NADH₂, the more negative couple reduces the less negative one under standard conditions. Given a supply of the reduced substrate of the more negative couple, the NAD/NADH₂ ratio is therefore bound to fall.

If, then, the redox potentials of the dehydrogenases present limit the possible degree of reduction of NAD, other factors determine the actual redox state within the permissible range. These include the concentrations of reduced and oxidized substrates of dehydrogenases; the rate of removal of NADH₂ by the respiratory chain, which in turn is controlled by the concentrations of ADP and Pi, and by substrates which join the chain at the flavoprotein level (succinate, fatty acyl-CoA esters); and the rate of transfer of reducing equivalents between cytoplasm and mitochondria by the malate and α -glycerophosphate shuttles [see (13)].

The redox state of the NADP couple.—Preliminary assays of the redox state of the NADP couple, based on the malate enzyme of the cytoplasm, indicate that in the normal, well-fed rat liver the value of the NADP/NADPH₂ ratio is of the order of 1/240, which differs from the NAD/NADH₂ ratio for this compartment by a factor of 174,000. Such a difference is possible because cytoplasm does not contain transhydrogenases. As for the mitochondria, the very high activity of the mitochondrial glutamate dehydrogenase capable of reacting with both NAD and NADP makes it highly probable that the mitochondrial NAD and NADP couples are in equilibrium. If this is the case, the NADP/NADPH₂ ratio would be 1.5 times the value of the NAD/NADH₂ ratio; *i.e.*, it would be about 11.4 in the mitochondria of the well-fed rat liver.

RESUMEN

Se midieron las concentraciones de los sustratos oxidados y reducidos de los sistemas de deshidrogenasas de lactato, β -hidroxibutirato y glutamato, en hígado de rata ligados y congelados lo más pronto posible después del sacrificio del animal. Los sustratos de estas deshidrogenasas están casi en equilibrio con NAD y NADH₂ libres, y la relación de las concentraciones de los dinucleótidos libres se pueden

calcular, por lo tanto, a partir de las concentraciones de los sustratos medidas experimentalmente y de las constantes de equilibrio. El sistema de la deshidrogenasa láctica refleja la relación NAD/NADH_2 en el citoplasma, mientras que el sistema de deshidrogenasa de β -hidroxibutirato y el de glutamato reflejan esta relación en las crestas mitocondriales y en la matriz mitocondrial, respectivamente.

La relación promedio NAD/NADH_2 a pH 7.0 del citoplasma del hígado de rata se calculó en 725 para ratas bien alimentadas, en 528 para las ratas en inanición y en 208 para ratas diabéticas por loxana.

Las relaciones NAD/NADH_2 para la matriz y las crestas mitocondriales dieron prácticamente idénticos resultados en el mismo estado metabólico. Esto indica que las dishidrogenasas de β -hidroxibutirato y de glutamato comparten una "poza" común del dinucleótido. La relación promedio NAD/NADH_2 en el interior de las mitocondrias del hígado de ratas bien alimentadas fue de cerca de 8; bajó a 5 en la inanición y se elevó a unos 0 en la diabetes aloxánica. Las relaciones NAD/NADH_2 del citoplasma y de la mitocondria son, por lo tanto, muy diferentes y no cambian de manera paralela cuando el estado metabólico del hígado se altera.

Se discuten las implicaciones de estos hallazgos sobre diversos problemas que comprenden los siguientes: número de "pozas" de $\text{NAD}-\text{NADH}_2$ en las células hepáticas; la aplicabilidad del método usado para otros tejidos distintos del hígado de rata; el significado fisiológico de las diferencias de los estados redox de la mitocondria y del citoplasma; los aspectos de la regulación del estado redox en los compartimentos celulares y las relaciones entre el estado redox de los compartimentos celulares y la cetosis.

Se proporcionan datos provisionales sobre el estado redox de la pareja NADP en el hígado de rata.

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